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Hormonal Feminization and Associated Reproductive Impacts in the Eastern Mosquitofish *Gambusia holbrooki*

Lokman Nor Hakim Norazmi
B. Sc. Agrotech (Aquaculture), M. Sc. (Aquaculture)

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Doctor of Philosophy

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The following people and institution contributed for the publication of work undertaken as part of this thesis:

Primary Author:

Lokman Nor Hakim Norazmi
Institute for Marine and Antarctic Studies, University of Tasmania.

Co-Authors:

G. John Purser
Institute for Marine and Antarctic Studies, University of Tasmania.

Jawahar G. Patil
Institute for Marine and Antarctic Studies, University of Tasmania.
Inland Fisheries Service, Tasmania.

Candidate was the primary author. Co-authors contributed to the idea, its development and assisted with the refinement and presentation of the work.

I, the undersigned, agree with the stated proportion of work undertaken for the published peer-reviewed manuscript contributing to this thesis.

Associate Prof. John Purser	**Prof. Chris Carter**
Primary Supervisor	Head, Fisheries and Aquaculture Centre
Institute for Marine and Antarctic Studies, University of Tasmania	Institute for Marine and Antarctic Studies, University of Tasmania
(Date: 28th June 2016)	(Date: 28th June 2016)

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Lokman Nor Hakim Norazmi

(Date: 28th June 2016)

Dedication

To my late mother… for she is the main reason I am here.

Rohani Said

15th June 1958 – 11th December 2000

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Amazing | Humbled | Growth | Inspired | Blessed

It has been a wild rollercoaster ride, but these five words perfectly described my Ph.D. adventure in this beautiful island state. Though some might say that the Ph.D. journey is a lonely one, never have I felt alone throughout my adventure due to the presence of the following individuals and institutions that have made me the person I am today.

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Abstract

The eastern mosquitofish *Gambusia holbrooki* has been listed in Australia as a noxious invasive species that requires control and eradication solutions on a large spatial scale. The Trojan Sex Chromosome (TSC) strategy has been proposed in recent years as a new genetic solution to control invasive aquatic species. The TSC approach involves the release of Trojan chromosome carriers, individuals produced via hormone sex reversal into the wild to skew the sex ratio of the population. As a first step towards applying the TSC strategy in *G. holbrooki*, this study systematically investigated and documented the relationship of the gravid spot with gestation, clutch size and timing of parturition – knowledge that are essential for efficient administration of hormones in this species. Importantly, by utilizing this new knowledge, the efficacy of two feminizing hormones (Diethylstilbestrol-DES and Estradiol-E2) was tested and the reproductive fitness of treated fish assessed.

The study identified the gravid spot of females as an excellent marker to predict embryonic developmental progress and reproductive output in *G. holbrooki* by means of quantifying its visual attributes (intensity and size). An equation to predict clutch size using the relationship between gravid spot intensity and size together with fish length was ascertained [$CS = 1.835 - (0.85\ X\ SS) + (0.196\ X\ SI) + (3.543\ X\ FL)$, where SS and SI are gravid spot size and intensity respectively, and FL is fish length]. The reliability of these findings was confirmed when it facilitated design and accurate observation on *G. holbrooki* gestation period and parturition behaviour. The gestation was significantly longer ($F=364.58$; $df=1,48$; $P>0.05$) when reared at 23°C (39±1.91 days) compared to 25 °C (28.6±1.94 days). However, temperature did not have

significant impact (P>0.05) on clutch size or diel timing of parturition which occurs predominantly in the morning (0900-1100h). The first-ever description on the posture of *G. holbrooki* fry (progenies) during parturition was also reported where the tail of the fry emerged first with a few exceptions of head-first, twin and premature births. All this information especially the utility of the gravid spot as an external marker of embryonic development was used to structure the sex reversal experiments.

Sex reversal experiments administering both DES and E2 through food targeted two life stages separately: (i) embryonic stage through gravid females and (ii) newborn juveniles. The concentrations of DES tested at both life stages ranged between 20 to 100 mg/kg feed. Two control groups were set for each experiment: (C1) normal feed (no chemical exposure) and (C2) feed mixed with 70% ethanol (vehicle control). In the first experiment, DES treatment did not affect the duration of gestation period in parturating females but as the treatment continued, DES was found responsible for gonadal atrophy in all the treated females after 30 days of exposure. The mean survival rate (MSR) of juveniles was significantly lower ($F=16.79$; df: 6, 15; $P<0.05$) compared to the controls (C1: 74.76±15.37%; C2: 71.26±18.5%) where the highest MSR was only 30.15±6.05% in fish treated at the concentration of 40 mg/kg feed for the duration of 11-23 days. Similarly, the MSR of juveniles fed with DES supplemented diet (second experiment) was also significantly lower ($F=3.216$; df: 6, 28; $P<0.05$) than controls (C1: 78.42 ±22.0%; C2: 73.8 ±20.22%) with the highest MSR shown by fish treated with DES at 20 mg/kg feed for 30 days (45.6 ±17.93%). Unexpectedly, masculinization was observed in all treated fish (in both experiments 1 and 2) where all the juveniles were phenotypically (morphologically) male based on the elongation of the anal fin. Observation of treated fish at 365 days after parturition (DAP)

old revealed that all individuals possessed an under-developed gonopodium compared to normal males, suggesting a paradoxical masculinising and an incomplete sex-reversal effect on genetic females and males respectively in all the treated groups. This condition also seems to have affected the reproductive viability of the fish since none managed to breed with unexposed fish. A paradoxical effect even at relatively low doses of treatment suggests that DES is not a suitable feminising agent for *G. holbrooki* sex reversal. Molecular and cellular experimentation is warranted for further understanding of the mechanism underpinning this rare observation.

Similar to DES treatment, E2 was administered at concentration ranging between 50-400 mg/kg of feed in two life stages —embryos and juveniles. Two control groups were also set as those in the DES experiment. The MSR of controls were found to be slightly higher ($F=4.38$; df: 6, 27; $P<0.05$) compared to the treatment groups (C1: 79.96±20.327%; C2: 77.09±10.32%). Treatment of E2 at 200-400 mg/kg feed between 12-21 days successfully produced a 100% female population in the embryo administered group, with the highest MSR of 59.33±12.54% shown by those treated at 200 mg/kg feed. The gestation period of the treated females was not altered by the exposure to E2. In the second group (juvenile administration), the MSR of controls were also significantly higher ($F=7.27$; df: 5, 24; $P<0.05$) compared to the treatment groups (C1: 71.73±22.86%; C2: 70.02±18.26%). A 100% feminization was achieved at all administered doses with juveniles treated with an E2 concentration of 50 mg/kg feed displaying the highest MSR at 66.38±12.34%. The survival rates observed in this study are substantially low compared to other E2 treated livebearers such as guppies and black mollies. High stocking densities which lead to aggression by dominant females is proposed as one of the reasons for the low survival rates in E2 treated *G. holbrooki*.

Reproductive fitness assessment was conducted on two groups of *G. holbrooki* that showed the best performance (highest MSR and feminization percentage) in the sex reversal experiment namely: (i) fish that were treated with E2 at 200 mg/kg of feed (first experimental group); (ii) those exposed to E2 concentration of 50 mg/kg of feed (second experimental group). In parallel a control group (unexposed fish) was also assessed. All juveniles were reared to maturity and bred with normal males. In general the assessment shows that E2 treatments at optimum dose did not compromise the reproductive fitness of the treated fish. There was no significant difference ($P>0.05$) in terms of ability to breed, gestation period, clutch size and the MSR of progenies produced between treated fish and controls. These observations demonstrate that the reproductive fitness of E2 treated fish is on par with controls. Nevertheless, the number of progeny produced by females in all three groups was low (1-5 fish) warranting continued long-term observations of subsequent clutches.

In conclusion, feminization of *G. holbrooki* was successfully achieved by using E2 as a feminizing agent. The study established a protocol to successfully feminize this species at optimum dose of E2 through oral administration either during the embryonic stage via female brood (200 mg/kg) or to newborn juveniles (50 mg/kg). The protocols and information generated in this study provide a basis for further refinement of hormone treatment and for developing the TSC strategy to control and eradicate this noxious pest. Other studies on fish reproductive biology and ecology especially in livebearing species as well as research on ecotoxicology and pest fish management will benefit from the outcomes presented in this thesis.

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List of Abbreviations

IAS	Invasive Alien Species
IUCN	International Union for the Conservation of Nature
USA	United States of America
NRM	Natural Resource Management
TSC	Trojan Sex Chromosome
EDC	Endocrine Disrupting Compound
DES	Diethylstilbestrol
E1	Estrone
E2	Estradiol
EE2	17α-ethynylestradiol
MSR	Mean Survival Rate
AEC	Animal Ethics Committee
SL	Standard Length
TL	Total Length
ml	Millilitre
mg	Milligram
mm	Millimetre
cm	Centimetre
kg	Kilogram
IBM	International Business Machines
SPSS	Statistical Package for the Social Sciences

ANOVA	Analysis of Variance
DAP	Days After Parturition
RAS	Recirculating Aquaculture System
EtOH	Ethanol
GnRH	Gonadotrophin releasing hormone
GtH	Gonadotrophin hormones
LH	Luteinizing hormone
FSH	Follicle stimulating hormone
Vtg	Vitellogenin
MT	Methyltestosterone

CHAPTER 1

General Introduction and Research Aims

1.1. Invasive Alien Species

The European Union defines an invasive alien species (IAS) as any non-native species that poses threats upon its introduction and/or spread outside their natural past or present ranges (Kettunen et al., 2008). The spread and establishment of IAS has increased at an alarming rate in recent years thus becoming a major environmental issue due to its destructive impact on biodiversity on a global level. Identified as the second major threat to biodiversity after habitat destruction (Kettunen et al., 2008, Environment and Communications References Committee, 2015), IAS also have a significant negative impact on the economy of a nation (Touza et al., 2007, Fitzgerald and Wilkinson, 2009, Gong et al., 2009). In the United States alone, the total losses due to IAS have accumulated to almost USD120 billion per year (Pimentel et al., 2007). In Australia, the estimated value of economic loss in the agriculture sector is at AUD743.5 million per annum and this only accounts for the mitigation cost of exotic pest species which includes foxes, rabbits, wild dogs, pigs, mice and pest birds (Gregory et al., 2014).

It is estimated that there are more than 4000 IAS in Australia, including plants, vertebrates, invertebrates and microorganisms (Nentwig, 2008), of which 180 are alien fish species. According to several reports, they were introduced to Australia via ornamental releases, species acclimatization trials, ballast water, aquaculture and pest bio-control (Koehn and MacKenzie, 2004, Lintermans, 2004). The International Union for the Conservation of Nature (IUCN) listed 8 species as the worst aquatic IAS globally (Lowe et al., 2000), five of which are here in Australia. They are: brown trout (*Salmo trutta*), common carp (*Cyprinus carpio*), Mozambique tilapia (*Oreochromis mossambicus*), rainbow trout (*Oncorhynchus mykiss*) and also the eastern mosquitofish (*Gambusia holbrooki*) (Koehn and McDowall, 2004).

1.2. The Eastern Mosquitofish *Gambusia holbrooki*

Native to North America, this small freshwater teleost belongs to the poeciliidae family. It is often confused with the western mosquitofish, *G. affinis* due to their similarity in appearance and biology. Both species are generically known as Gambusia but other common names includes Top Minnow and Plague Minnow (Pyke, 2005). Initially regarded as worthless and of no importance (hence its Latin name), Gambusia has become the most widespread freshwater species being found in all parts of the world except Antarctica. Its spread was driven by its popularity as a mosquito control agent, hence the name mosquitofish (Krumholz, 1948, Lloyd and Tomasov, 1985, Pyke, 2005). In recent years, the former common name (Gambusia) is preferred due to its negative impact on native fauna and flora and also to change public perception on its use as a mosquito control agent.

1.2.1. Biology of *Gambusia holbrooki*

Generally, the size of Gambusia range between 1.0-8.0 cm where the maximum size of females can reach up to 8.0 cm while the males are about 3.5 cm (Vondracek et al., 1988, Pyke, 2005, Froese and Pauly, 2015). It has been reported that all Gambusia males and most females live less than 12 months and few females live up to three years, however their longevity in the wild rarely exceeds 12-15 months (Vargas and de Sostoa, 1996, Pyke, 2008). Known to be omnivorous, their diet in the wild varies from animal to plant materials consisting of worms, insects (including terrestrials insect such as ants), small fish, tadpoles and phytoplankton (Pyke, 2005).

The diet of Gambusia indicates that its microhabitat is near the water surface (García-Berthou, 1999). Gambusia prefers warm (31-35°C), shallow (8-15 cm deep), still and slow moving water as habitats. Being a widespread species, they are known to be very tolerant of and have a high adaptability to a wide range of environmental conditions. They can be found in undisturbed aquatic environments such as lakes, swamps, wetlands and streams to those of extreme conditions like urban drains and polluted water bodies. Various reports have shown that Gambusia can withstand water temperature between 4-44°C (Al-Johany and Yousuf, 1993, Pyke, 2005), salinity up to 58.2 parts per thousand (ppt) (Morgan et al., 2004), pH ranging from 4.5-9.0 (Keup and Bayless, 1964) and dissolved oxygen around 1-11 mgL^{-1} (Odum and Caldwell, 1955, Cherry et al., 1976). The ability of Gambusia to tolerate broad environmental conditions has facilitated its survival and widespread population establishment worldwide.

The life stages of this livebearer can be divided into three phases; i) embryonic; ii) juvenile/immature and iii) adult/mature (Pyke, 2005). The duration of the first two life stage phases depends on temperature, seasons and locality. For example, the embryonic life phase also known as the gestation period normally ranges between 22-25 days at the optimum temperature of 25-26 °C but can extend up to 50 days at a lower temperature (Krumholz, 1948, Reznick, 1981, Milton and Arthington, 1983, Keane and Neira, 2004). Males usually mature earlier than females. For males, the immature phase period lasts between 18 days to 8 weeks while in females it extends between 18 days to 10 weeks (Vondracek et al., 1988, Meffe, 1992, Koya et al., 2003). However, this life stage duration can last up to 8 months for both sexes if the water temperature is below the optimum level (Trendall, 1982, Vondracek et al., 1988).

A male is considered mature when it has a fully developed gonopodium, a modified structure of the anal fin used during mating to transfer sperm to females (Pyke, 2005). In females, a dark gravid spot on both sides of its belly slightly anterior to the anal pore will develop when it matures (Kristensen et al., 2007, Pyke, 2008). The annual breeding season of Gambusia runs between mid-spring to mid-autumn and peaks during summer (Keane and Neira, 2004, Pyke, 2005). Unfortunately, no information can be found on its reproductive cycle at tropical geographic locations. The females are capable of parturating up to nine clutches of live young per year with each clutch size varying from one to over 300 fry (Krumholz, 1948, Gall et al., 1980, Trendall, 1982, Milton and Arthington, 1983, Keane and Neira, 2004, Pyke, 2005). The sex ratio of the juveniles at birth is reported to be 1 male: 1 female and is not influenced by the environment (temperature/photoperiod) (Krumholz, 1948, Vargas and de Sostoa, 1996).

As in other poeciliids, fertilization occurs internally in this species (Constantz, 1989). During mating, the male will transfer the sperm to the female by swimming closely from behind and inserting its gonopodium into the genital opening of the female (Peden, 1972). The sperm is retained in an intra-ovarian structure known as the ‘delle’ and reports have shown that female Gambusia have the ability to store sperm from multiple inseminations (including multiple mates) for a few months (Chesser et al., 1984, Robbins et al., 1987, Koya et al., 2000). Fully mature oocytes are fertilized within the ovary by the stored spermatozoa and the embryos develop in the ovarian follicles throughout the gestation period before being born as juveniles (Bone and Moore, 2007). The fertilization of the next clutch is thought to occur after the birth of the previous clutch (Koya et al., 2000). The detailed embryonic life stages of Gambusia have been described in *G. affinis* (Chambolle et al., 1970, Koya et al., 2000) but not in *G. holbrooki*.

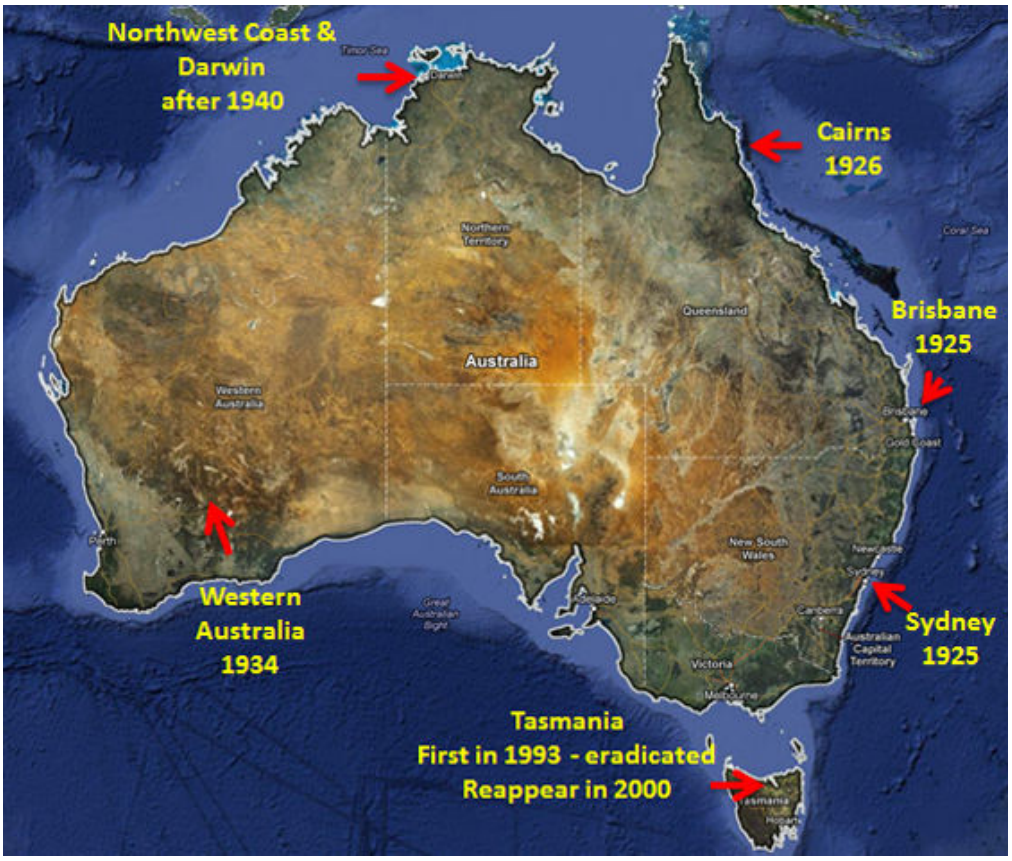
Although *G. holbrooki* and *G. affinis* are closely related and *G. holbrooki* was once considered a subspecies of *G. affinis* (Wooten et al., 1988, Rauchenberger, 1989), both species differ on the basis of their meristic and genetic characteristics as well as gonopodium structure and chromosome morphology. Meristically, they can be distinguished through the number of dorsal and anal fin rays. *Gambusia holbrooki* possesses seven dorsal fin rays and 10 anal fin rays while *G. affinis* possesses only six dorsal and nine anal fin rays (Lloyd and Tomasov, 1985). In terms of genetic characteristics, both species are reported to have different patterns of allele frequencies and abrupt differentiation in local genetic constituency (Wooten et al., 1988). Males of both species also have a distinct gonopodial characters at the third and fourth ray. In *G. holbrooki*, the third ray of its gonopodium has a series of prominent denticles while the fourth ray consists of unsegmented bony claw which is in contrast to *G. affinis* as its third ray lacks the denticles and the fourth ray has a segmented claw (Rosen and Gordon, 1951, Rosen and Bailey, 1963). While both species possess 24 chromosome pairs, the sex determining mechanism of *G. holbrooki* is distinctly different from *G. affinis* (Black and Howell, 1979, Angus, 1989a). *Gambusia affinis* demonstrates a chromosomal heteromorphy with a WZ-ZZ sex determining mechanism (Chen and Ebeling, 1968, Black and Howell, 1979) but no evidence of heteromorphy has been found in G. *holbrooki*. Assumption that the sex determination mechanism in this species is XX-XY was based on the sex-linked inheritance of melanistic pigmentation in males (Angus, 1989a, Angus, 1989b, Horth, 2006, Horth et al., 2013). However, there is still some confusion where recent reports continue to assume that both species share the same gamity of WZ-ZZ (Thresher et al., 2013).

1.2.2. *Gambusia holbrooki* in Australia and Tasmania

As mentioned earlier, *G. holbrooki* and *G. affinis* are often confused as the same species due to their similarity. Even in Australia, prior to 1985, the populations of Gambusia were thought to be of *G. affinis*, the same as those in New Zealand. However, studies by Milton and Arthington (1983) and Lloyd and Tomasov (1985) have demonstrated that the populations of Gambusia in Australia consist of only *G. holbrooki*. The translocation pathway of the Australian Gambusia population involved fish taken from Georgia, USA (natural locality of *G. holbrooki*) and brought to Italy before extending to other locations in Europe and Australia while those in New Zealand originated from the Texas, USA population (natural locality of *G. affinis*) that were distributed to Hawaii and other Pacific islands including New Zealand (Pyke, 2005, Pyke, 2008, Vera et al., 2016).

Gambusia holbrooki was first introduced to Australia in 1925 as a biological control for mosquitoes where the first batch was released at the Botanical Gardens in Sydney, New South Wales (NSW National Parks and Wildlife Service, 2003). Since then, they have spread all over the country especially during World War II by military and local health authorities and also during the 1974 floods (Rowe et al., 2008). The occurrence of Gambusia in Tasmania was first recorded in 1992 in the northern part of the state but fish were successfully eradicated by state government agencies (Lynch, 2008). However, they reappeared at the Tamar River Wetlands area in Launceston, North of Tasmania in 2000 and in 2006 a small population of Gambusia was reported in a few ponds in the southern part of the state (Hardie et al., 2006, Lynch, 2008). Figure 1.1 shows the history of introductions (Fig. 1.1A) and the current distribution of this species in Australia (Fig. 1.1B).

(A)



(B)

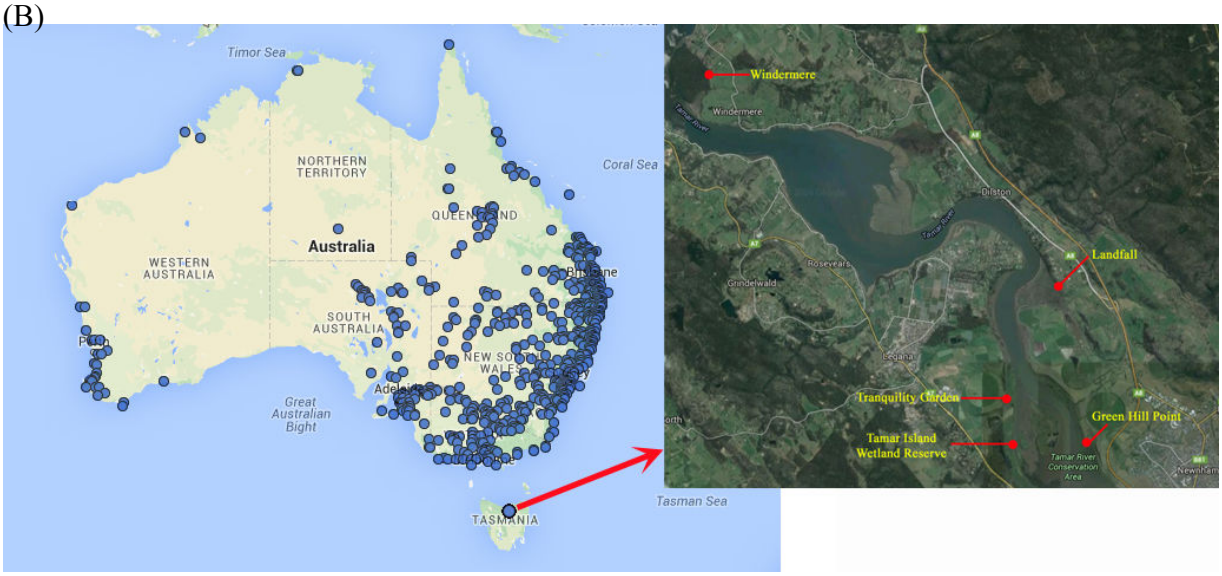


Fig. 1.1. Map of Australia showing (A) Introduction history and (B) Current distribution of *G. holbrooki* in Australia. The location of *G. holbrooki* population in Tasmania is shown in the inset. (Maps adopted and modified based on Rowe et al. (2008), Lynch (2008), Patil (2012), Online Zoological Collections of Australian Museums (2015) and NRM North (2015)).

1.2.3. Impact of *Gambusia holbrooki* in Australia

The impact of Gambusia on native environments has been comprehensively studied both in the field and in laboratories. Examples of the impacts of this species on local fauna include predation, intense competition for food and habitat, modification of habitat and interference with reproduction (Ivantsoff and Aarn, 1999, Pyke, 2005, Keller and Brown, 2008). Gambusia attack and show aggressive behavior towards native tadpoles and fish, for example by nipping the fins of tadpoles and galaxiids (Komak and Crossland, 2000, Baker et al., 2004). In addition to fish and frogs, piscivorous water birds are also affected by this invasive species. A study at Manly Lagoon, Sydney found traces of bio-accumulated zinc at very high concentrations in a high density population of Gambusia potentially affecting fish and water bird species that may predate on them (Van den Broek et al., 2002). In terms of disease and parasite transmission, Gambusia is recognized as a vector of the exotic Asian fish tapeworm that affects native fish (Arthington et al., 1989, Dove and Fletcher, 2000).

Gambusia is currently threatening at least 23 native fish species and is responsible for the depletion of a least 15 frog species (Rowe et al., 2008). Some of the native fish species includes the red-finned blue-eye, *Mogurnda adspersa* (Warburton and Madden, 2003), native galaxiids, *Galaxias sp*. (Becker et al., 2005, Slinger, 2012) and gudgeons, *Hypseleotris sp.* (Arthington and Marshall, 1999, Arthington et al., 1983) while frog species include the green and golden bell frog, *Litorea aurea* (Pyke and White, 2000, Hamer et al., 2002) and the ornate burrowing frog, *Limnodynastes ornatus* (Komak and Crossland, 2000). A more complete list of threatened native fish and frogs is shown in Table 1.1.

Table 1.1. List of native fish and frog species that are currently threatened by Gambusia (based on Rowe et al. (2008)).

Fauna	Common name	Scientific Name	Type of Study	
			Field-based	Lab-based
Fish	Agassiz's glassfish, olive perchlet	*Ambassis agassizi*	*	*
	Crimson-spotted rainbow fish	*Melanotaenia duboulayi*	*	*
	Purple-spotted gudgeon	*Mogurnda adspersa*	*	
	Pacific blue-eye	*Pseudomugil signifer*	*	*
	Australian smelt	*Retropinna semoni*	*	
	Red-finned blue-eye	*Scaturiginichthys vermeilipinnis*	*	
	Firetail gudgeon	*Hypseleotris galii*	*	*
	Empire gudgeon	*Hypseleotris compressa*	*	
	Midgley's carp gudgeon	*Hypseleotris sp. 1*	*	
	Western pygmy perch	*Nannoperca vittata*	*	*
	Nightfish	*Bostockia porosa*	*	
	Ornate rainbowfish	*Rhadinocentrus ornatus*	*	*
	Oxleyan pygmy perch	*Nannoperca oxleyana*		*
	Southern pygmy perch	*Nannoperca australis*	*	*
	Common jollytail	*Galaxias maculatus*		*
	Dwarf galaxias	*Galaxias parvus*	*	
	Western minnow	*Galaxias occidentalis*	*	
	Eastern little galaxias	*Galaxiella pusilla*	*	*
	Black-stripe minnow	*Galaxiella nigrostriata*		*
	Edgbaston goby	*Chlamydogobius squamigenus*	*	
	Desert goby	*Chlamydogobius eremius*	*	
	Spangled perch	*Leiopotherapon unicolor*	*	
	Murchison River hardyhead	*Craterocephalus cuneiceps*	*	
Frogs	Common froglet	*Common froglet*		*
	Sign-bearing froglet	*Crinia insignifera*	*	*
	Glauert's froglet	*Crinia glauerti*		*
	Tschudi's froglet	*Crinia georgiana*		*
	Green and golden bell frog	*Litoria aurea*	*	*
	Lesueur's frog	*Litoria lesueuri*		*
	Bleating tree frog	*Litoria dentata*		*
	Slender tree frog	*Litoria adelaidensis*		*
	Yellow-spotted tree frog	*Litoria flavipunctata*	*	
	Southern brown tree frog	*Litoria ewingii*	*	
	Cooloola sedge or tree frog	*Litoria coolooensis*	*	
	Spotted marsh (grass) frog	*Limnodynastes tasmaniensis*		*
	Striped marsh frog	*Limnodynastes peronii*		*
	Ornate burrowing frog	*Limnodynastes ornatus*		*
	Moaning frog	*Heleioporus eyrie*		*

1.2.4. Control and eradication of *Gambusia holbrooki*

The control of this aquatic pest in Australia has been attempted through legislation, policy, public awareness campaigns and building barrier structures at affected areas to stop it from spreading further (Lynch, 2008, Rowe et al., 2008). In the state of Tasmania for example, the Inland Fisheries Act 1995 prohibits the fish from being imported, moved or kept in the state where heavy fines await those who break the law (Inland Fisheries Service, 2014) while organizations such as the Natural Resource Management play their role by conducting activities such as school engagement programs, television commercials and public displays and exhibitions to create public awareness of the destructive impact of Gambusia (Scurr et al., 2011, NRM North, 2015).

At present, the strategies for the eradication of this species can be divided into two main approaches: physical and chemical. In the physical approach, trapping and netting were employed and have shown to be quite effective but it requires constant effort to ensure significant effect can be seen (Koehn and MacKenzie, 2004, Kerezsy, 2009). In Tasmania, a volunteer-based Gambusia trapping program led by NRM North, has been set-up and maintained as an on-going control effort for this pest fish (Scurr et al., 2011, NRM North, 2015). Research into Gambusia trap developments has also been undertaken by the Australian Maritime College, University of Tasmania. The research involves developing thermal trapping gear equipped with lights and attractants that has the ability to control surrounding water temperature to specifically attract and trap Gambusia (Scurr et al., 2011). However, problems such as power usage hinder further development of the trapping device. For the second approach, chemicals such as rotenone and liquid chlorine have been used in several attempts to eliminate this species and have shown

mixed results (Pyke, 2008, Kerezsy, 2009). The negative side of both approaches is that it also affects non-target native species where they are accidentally trapped and poisoned.

Few studies have been undertaken to find an alternative approach such as bio-control that is not only target-specific but also does not damage the environment. Bio-control may include several techniques. The first technique involves the introduction of predators. However, information on predators or potential predators in its invaded range is still lacking (Jackson and Bamford, 2011) although information on the predators of Gambusia in its native range is available (i.e.: wading birds and aquatic snakes) (Jenkins, 2011). Researchers in Australia have explored the potential of native species such as Australian bass (*Macquaria novemaculeata)* (Grigaltchik et al., 2012) and the introduced yabbies, *Cherax destructor* and *Cherax cainii* (Beatty, 2006) as predators for Gambusia but the predator-prey studies conducted shows that they are not highly effective. Contraceptive controls have also been explored as an alternative method of eradication but problems such as methods to disperse the immune-contraceptive drugs hampered its development (Rowe et al., 2008).

Many of these techniques currently either require further investigation or are less than ideal. It is apparent that a more effective method must be developed to control, eradicate and prevent the re-establishment of this pest fish (Gambusia) population in Tasmania and Australia in general. Recently, focus has been directed to using genetic control as an option for eradicating this noxious aquatic pest.

1.3. Trojan Sex Chromosome

According to Hamilton (1967), a skewed sex ratio could lead to the extinction of a population. This suggestion forms the basis of many modern genetic pest control strategies including the Trojan sex chromosome (TSC) approach (Gutierrez and Teem, 2006). Since then, the interest in this approach has grown significantly as is demonstrated by the growing literature on the topic (Appendix 1).

In the TSC approach, Trojan chromosome fish, individuals carrying sex chromosome with reversed phenotype (e.g. XY or YY females), would be released repeatedly to breed with the wild fish population. This will skew the sex ratios of the population thus leading to its extinction after several generations (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007, Stelkens and Wedekind, 2010). As an example, in the case of IAS with an XX-XY sex determination mechanism, the constant introduction of Trojan females, a YY chromosome female fish produced via hormone sex reversal (Fig. 1.2), will lead to a heavily male biased population (Fig. 1.3), in theory resulting in the extinction of the IAS in the wild. Although the TSC approach has not been tested in the field, a precedent case on environmentally sex reversed Chinook salmon (*Oncorhynchus tshawytcha*) population in the Columbia River, USA (Nagler et al., 2001) and a model-based study on environmental sex reversal in fish (Hurley et al., 2004) have shown that a skewed sex ratio caused by breeding sex reversed and normal individuals will lead to population collapse thus supporting the theory of this strategy. Few mathematical models that have been developed to investigate its effectiveness in IAS eradication programs including Gambusia, have also shown that this method can be effective to eradicate invasive unwanted pest fish (Bax and Thresher, 2009, Teem and Gutierrez, 2010, Parshad et al., 2010, Parshad and Gutierrez, 2011,

Gutierrez et al., 2012, Senior et al., 2013, Teem et al., 2014). Recently, works on the development of this strategy to eradicate unwanted brook trout (*Salvelinus fontinalis*) population have been reported (Schill et al., 2016).

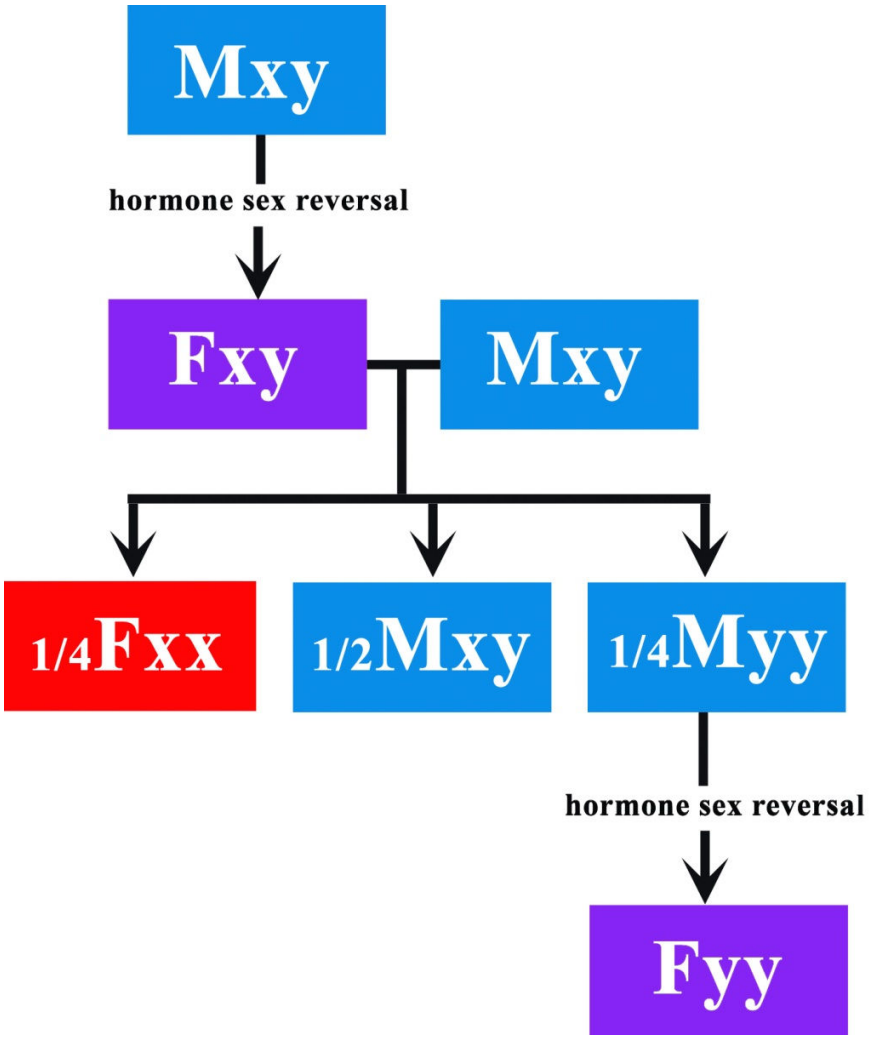


Fig. 1.2. Production of Trojan female fish, YY chromosome carrier individual, via hormone sex reversal (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007).

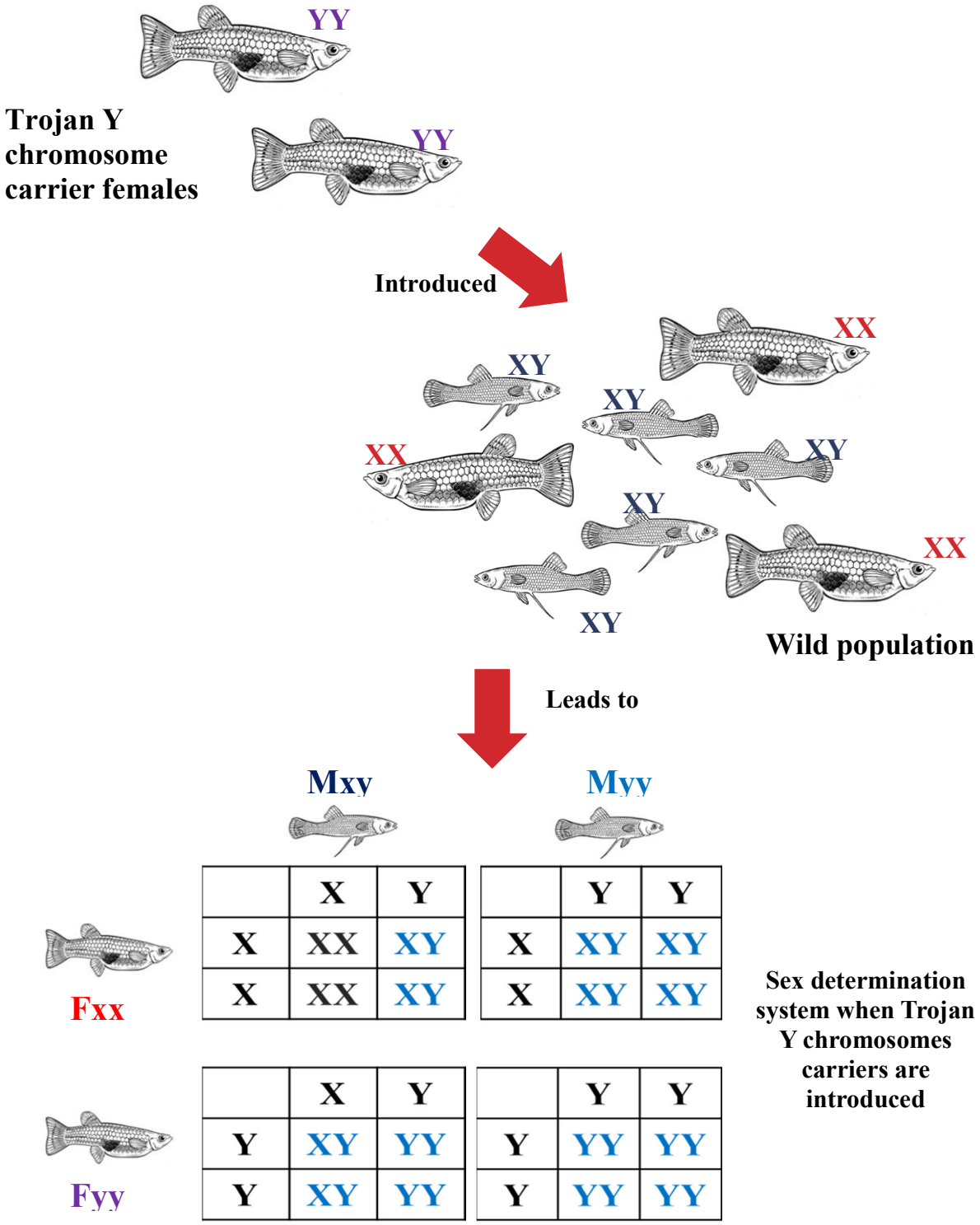


Fig. 1.3. Schematic showing outcome of Trojan sex chromosome approach in a fish species with XX-XY sex determination mechanism (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007).

There are a few advantages of TSC compared to other methods of IAS eradication. Firstly, TSC is species-specific which means it only affects the target IAS without harming native species (Gutierrez and Teem, 2006). Secondly, YY/ZZ individuals have been produced in a few species such as guppy (*Poecilia reticulata*) (Kavumpurath and Pandian, 1993b), black molly (*P, sphenops*) (George and Pandian, 1995), tilapia (*Oreochromis niloticus*) (Tuan et al., 1999) and even up until recently, female Trojan brook trout (*Salvelinus fontinalis*) (Schill et al., 2016) have successfully been developed therefore a general protocol for the production of Trojan individuals is available. Thirdly, Trojan carrier individuals can be produced through standard aquaculture practice without involving sophisticated DNA recombinant technologies that are laborious and expensive (Cotton and Wedekind, 2007). Finally, in the event of adverse effects, the TSC approach is reversible (Cotton and Wedekind, 2007).

Before the TSC can be applied in IAS eradication programs, certain requirements must first be met. These requirements are: (i) the gamity or the sex determination mechanism of the fish must be known; (ii) the target species must be susceptible to hormone sex reversal; (iii) the reproductive fitness of the chromosome carrier fish must be as efficient as wildtype (normal individuals) and (iv) the species must be amenable to mass propagation via aquaculture (Gutierrez and Teem, 2006, Teem and Gutierrez, 2010).

1.4. Hormone Sex Reversal in Fish

Unlike other vertebrates, the process of sex differentiation in teleosts is complex but labile thus making it possible to manipulate the sex of the fish through hormonal induction (Francis, 1992). The application of hormones to induce sex reversal in fish has come a long way since the 1930's; the sex reversal protocols have now been described for at least 56 species (Pandian and Sheela, 1995, Piferrer, 2001, Pandian and Kirankumar, 2003). For the fish biologist, hormonal sex reversal is a valuable tool to understand fish sex differentiation, biology and physiology while for the aquaculturist the process has been used for the production of monosex fish populations to improve commercial production and increase profits (Pandian and Sheela, 1995). Compared to other methods of sex control in fish, hormone sex reversal is widely used since it has a lower cost and it does not involve advanced technology that will then lead to the requirement of highly skilled labor (Piferrer and Lim, 1997).

The process of influencing sex differentiation to control the sex of the fish can be achieved by treating the fish with hormone during its labile period (Piferrer, 2001). At present, there are at least 31 natural or synthetic hormones available that have been used in sex reversal (Pandian and Sheela, 1995). A few examples of common hormones used in sex reversal include estradiol, testosterone, diethylstilbestrol (DES) and mibolerone (Pandian and Sheela, 1995). The former two are natural hormones whereas the latter are synthetic. The dose and duration of treatments varies between each hormone and fish species (Pandian and Sheela, 1995, Piferrer, 2001).

There are several methods by which the hormone can be administered: injections, implantation, orally via feeding and immersion (Piferrer, 2001). The latter two methods are commonly

preferred due to their effectiveness and ease of application to large numbers of individuals (Pandian and Kirankumar, 2003). Nonetheless, hormone administration protocols in terms of dose, window of lability and treatment period need to be identified and optimized for each species so that cost, time and effort can be minimized.

1.4.1. Hormone Sex Reversal in Poeciliids

Sex reversal protocols have been established in a number of the ornamental poecilids such as the swordtail (*Xiphophorus helleri*), molly (*Poecilia sphenops*) and guppy (*P. reticulata*) with the latter being the most extensively studied species (Piferrer, 2001). Hormone sex reversal is extensively studied in ornamental fish principally to produce male fish which display a much more attractive colour and pattern; producing a male-only population is thus much more profitable (Piferrer and Lim, 1997). In terms of steroids, estradiol is the most commonly used hormone in poeciliids followed by estrone, estradiol benzoate and DES; most were administered orally (Piferrer, 2001). There are three labile periods for hormone sex reversal in poeciliids: during embryogenic development (treatment through gravid females), post-parturition and post-maturity stages depending on species (Pandian and Sheela, 1995, George and Pandian, 1995, Piferrer, 2001). Typically, hormone treatment during embryogenic stages has been administered between 5-12 days before parturition while the treatment duration during post-parturition and post-maturity stages was between 7- 50 days (Kavumpurath and Pandian, 1992, Senior, 2013). Both labile periods and treatment durations are different between species in this family. By way of example, in *G. affinis*, treatment of estradiol at the concentration of 0.001 mg/L for 50 days on newborn juveniles (Senior, 2013) successfully produced sex reversed females while the same results were observed in guppy via the administration of 17α-ethynylestradiol orally at the dose

of 200 mg/kg food to gravid females 5-10 days prior to parturition (during embryogenic development) (Kavumpurath and Pandian, 1993b).

1.4.2. Hormone Sex Reversal in *Gambusia holbrooki*

To date, the only study conducted on hormone treatment to control sex differentiation in *G. holbrooki* was by Lepori in 1945 using oestrone (Piferrer, 2001). Since that report was published more than 50 years ago and in Italian, the process of obtaining detailed information on the dosage and protocols is problematic. The remainders of the studies on sex reversal in this species are restricted to exposure of juvenile and adult fish (post-parturition and post-maturity) to environmental pollutants that contain endocrine disrupting chemicals (EDC) (Table 1.2.). Here *G. holbrooki* is used as an indicator for pollution where the effects on secondary sexual characteristics, behavior and aromatase activity are monitored. However, none of these reports have reported the successful/complete sex reversal in this species. There is a general lack of information on the sex reversal in this species including protocols (dose, type of hormone, labile period and duration of treatment required) in contrast to the closely related *G. affinis* (Senior, 2013). For the present study, focus was directed to the feminization on *G. holbrooki*. Since production of Trojan chromosome carriers will involve fish feminisation (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007) based on the sex determination system possessed by this species (XX-XY) (Angus, 1989a, Angus, 1989b, Horth, 2006, Horth et al., 2013).

Table 1.2. List and effects of EDC's that have been studied in *G. holbrooki.*

EDC/Pollutant	Observed effects	Reference
Paper mill effluent	Female strongly masculinized displaying male physical secondary sex characteristics and reproductive behaviour while male displayed precocious development of physical secondary sex characters and reproductive behaviour	Howell et al. (1980)
	Females exhibit male secondary sex characters and behaviour. Both brain and ovarian aromatase activity where high in exposed females compared to unexposed ones thus suggesting the masculinization was due to androgenic contaminants.	Orlando et al. (2002)
	Females exhibit male secondary sex characters and behaviour.	Jenkins et al. (2003)
Kraft mill effluent	Female displayed masculinization of the anal fin.	Parks et al. (2001)
Municipal sewage effluent/Treated sewage effluent/Stormwater	Male gonopodium length reduced.	Batty and Lim (1999)
	Females exhibit male secondary sex characters and behaviour. No evidence of intersex fish was found based on gonad histological studies.	Leusch et al. (2006)
	Male gonopodium and anal fin length reduced.	Norris and Burgin (2010)
	Male gonopodium and anal fin length reduced.	Norris and Burgin (2011)
	Lower density and biomass of fish at polluted sites.	Midgley et al. (2014)
	Exposed males showed increased copulating behaviour compared to unexposed males. No sign of gonopodium alterations.	Saaristo et al. (2014)
4-Nonylphenol	Exposure to 50 μg/L on 3 days old juveniles for 75 days produced 100% female while exposure to 0.5 and 5.0 μg/L for the same duration produced individuals with incomplete gonopodium development. Gonad atrophy was	Drèze et al. (2000)

	also observed in all exposed males.	
Estradiol (E2)	Exposure on maturing juvenile males reduced its gonopodium length and lowers its sexual activity.	Doyle and Lim (2002)
	Exposure on adult males lower its sexual activity and they were less capable of impregnating females compared to unexposed males.	Doyle and Lim (2005)
	General delay in the development of three hemal spines.	Rawson et al. (2006)
17β-trenbolone	Female fish displayed elongated anal fin and vtg gene expression was reduced.	Brockmeier et al. (2013)
Unidentified pollutant containing EDC	Exposed male had slightly shorter gonopodia and fewer sperm cells	Toft et al. (2003)
	Decreased sperm count and sexual activity in exposed male.	Toft and Guillette (2005)
	Shorter gonopodia in exposed males.	Game et al. (2006)
	Females exposed to unknown EDC produce fewer but bigger embryos. Fecundity and E2 concentration in body tissues were not affected.	Kristensen et al. (2007)

1.4.3. Feminization Hormones

Feminization in fish has been extensively reviewed by Piferrer (2001). Fifteen different estrogenic substances (three natural and 12 synthetic) have been used to feminize fish (Pandian and Sheela, 1995, Piferrer, 2001) where the most common estrogens used (in no particular order) are estrone (E1), estradiol (E2), 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES) (Piferrer, 2001). Among these hormones, EE2 and DES are reported to be the most potent hormone where there were only little differences in the potency level between them (Piferrer, 2001). This is then followed by E2 and E1 (Piferrer, 2001). In this study, DES and E2 were chosen to be tested on *G. holbrooki*.

1.4.4. Diethylstilbestrol

DES was the first manufactured estrogen synthesized by scientists in 1938 (Dodds et al., 1938). It was prescribed to over 2 million pregnant women who were at risk of miscarriage between the 1940's to early 1970 and was taken off the market in 1971 when it was discovered it caused cancer (US Centre for Disease Control and Prevention, 2012, Wise et al., 2015). The first report on the use of DES to sex reverse fish was in 1953 where it was administered in medaka (*Oryzias latipes*) (Yamamoto, 1953). Since then it has been used to successfully sex reverse a number of fish species including the livebearing guppy (*Poecilia reticulata*) with the most usage in tilapias (*Oreochromis niloticus* and *O. mossambicus*) delivered via the feeding method (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b, Piferrer, 2001).

1.4.5. Estradiol

Estradiol (oestradiol or 17β-estradiol) is a female gonadal hormone but it is also present in males but in small quantities. Naturally, estradiol is synthesized by (i) the steroid producing cells located in the outer thecal and granulosa layers of the ovaries and (ii) the Leydig cell located among cysts of spermatogenic germ cells of the testis (Nakamura et al., 2003, Frisch, 2004). It can also be synthetically produced as a white crystalline powder. Estradiol is one of the first and the most widely used hormone in fish sex reversal where its first usage was reported in the early 1930's in guppy (*P. reticulata*) (Piferrer, 2001).

1.5. Research Aims

Complete hormone sex reversal has never been reported in *G. holbrooki*. There is no information available on hormone suitability and optimum dose required to sex reverse this fish. The most labile period for hormone treatment is also unknown let alone the reproductive fitness of treated fish. Furthermore, information on how to choose suitable gravid females for hormone treatment, gestation period in captivity and rearing protocol is still lacking. In this context, the main aim of this study was to develop a protocol to feminize this fish by gathering critical basic information on its reproduction, establishing rearing protocol and assessing the feasibility of hormone sex reversal in *G. holbrooki* including evaluating the reproductive fitness of treated fish and progeny. An outline of the experiments conducted is presented in section 1.6.

1.6. Thesis Structure and Chapter Summaries

Altogether there are four experimental chapters in this thesis (Chapter 2-4). Each chapter has been written as a stand-alone paper so that it can be published independently. The first experimental chapter (Chapter 2) has been published and the contributions of each author are as mentioned in the statement of co-authorship section at the beginning of this thesis. Due to the manner in which this thesis is written, there are unavoidable repetitions between each chapter however some extensive repetition in Chapters 3 and 4 have been consolidated. Fig. 1.4 illustrates the workflow of all the experiments and divisions of experimental chapters followed by the summary of each experimental chapter.

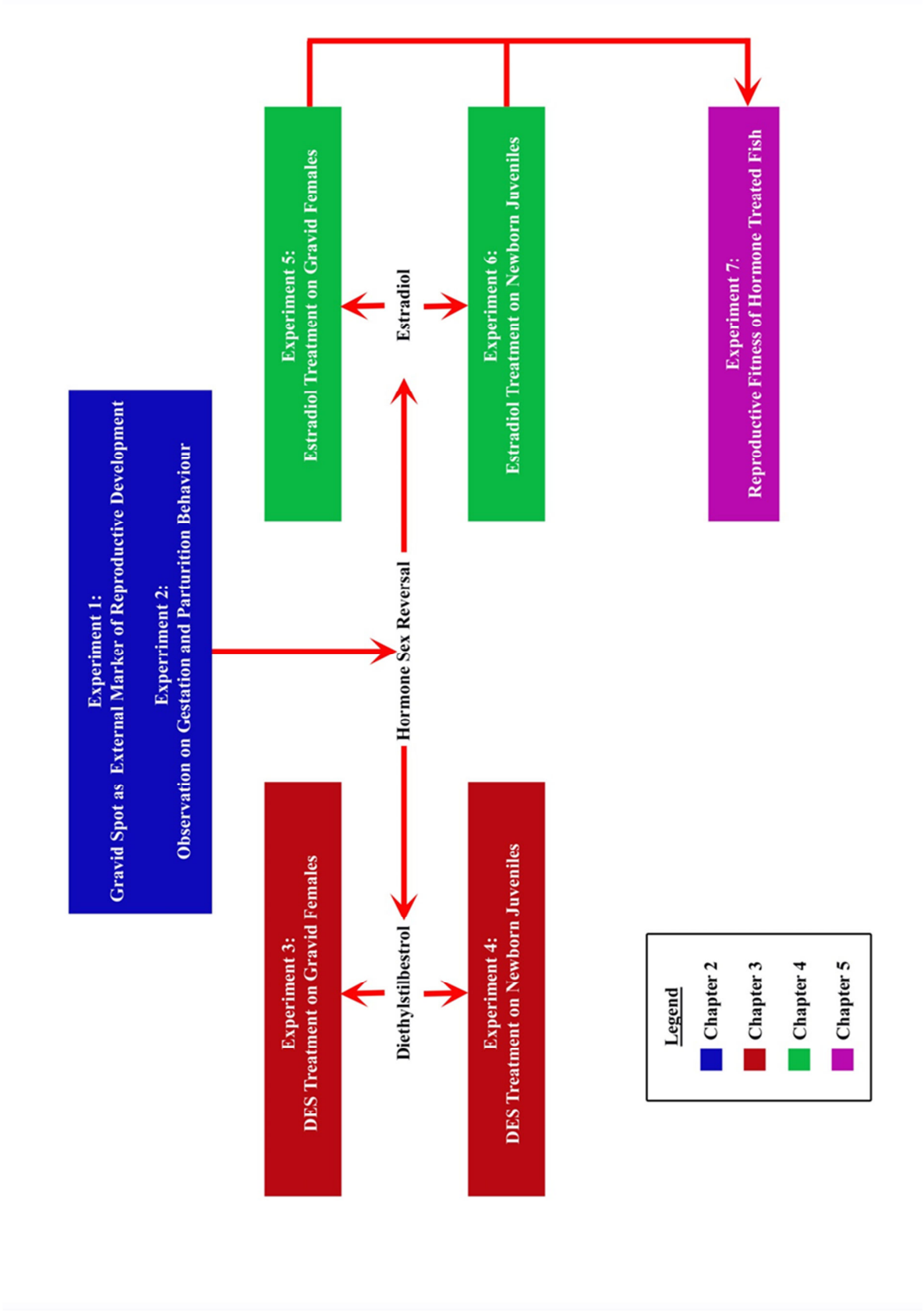


Fig. 1.4. Workflow of experiments and divisions of experimental chapters.

Chapter 2: GRAVID SPOT PREDICTS DEVELOPMENTAL PROGRESS AND REPRODUCTIVE OUTPUT IN A LIVEBEARING FISH, *Gambusia holbrooki*

The main aim of this chapter was to explore the use of the gravid spot of *G. holbrooki* as an external marker to predict the stage of oocyte and embryonic development, and reproductive output (Experiment 1). This was then followed by an observation of the gestation period and parturition behavior to test the robustness/plasticity of the relationship of the gravid spot and the developmental progress of this species (Experiment 2). The novel information obtained in this experimental chapter was critical for designing and conducting the sex reversal experiments in Chapters 3 & 4 i.e. to select females at similar embryonic developmental stages for hormone treatment, to know the precise time to start treatment on gravid females (by knowing the gestation period) and to optimize rearing protocols.

Chapter 3: PARADOXICAL EFFECT AND GONADAL ATROPHY IN THE EASTERN MOSQUITOFISH (*Gambusia holbrooki*) TREATED WITH DIETHYLSTILBESTROL (DES)

In this chapter, DES was administered orally on *G. holbrooki* at two targeted life stages separately: (i) embryonic stage through gravid females (Experiment 3) and (ii) newborn juveniles (Experiment 4). The concentrations of DES at both life stages ranged between 20 to 100 mg/kg of feed. Two control groups were set for each experiment: (C1) normal feed (no chemical exposure) and (C2) feed mixed with 70% ethanol (vehicle control). The effects of DES on the duration of gestation, survival rate and sex ratio of *G. holbrooki* were assessed. Observation was also conducted on the gonad of treated gravid females in Experiment 3 and anal fin of treated fish (progenies).

Chapter 4: EFFICACY OF ESTRADIOL ON SEX REVERSAL IN THE EASTERN MOSQUITOFISH (*Gambusia holbrooki*)

The efficacy of E2 to feminize *G. holbrooki* was assessed in this chapter. As in chapter 3, estradiol was administered orally on two separate life stage group (experiment 5 and 6) and two control groups were also set. However, in this chapter, the concentration of E2 was set between 50-400 mg/kg of feed. The sex ratio and survival rate of treated fish (progenies) along with the duration of gestation in treated gravid females were assessed and compared against controls.

Chapter 5: REPRODUCTIVE FITNESS OF THE EASTERN MOSQUITOFISH *Gambusia holbrooki* TREATED WITH ESTRADIOL

In this final experimental chapter, fish treated with estradiol (in chapter 4) that showed the best performance (highest MSR and feminization percentage) were bred and their reproductive fitness was systematically assessed against unexposed females (Experiment 7). Assessment was conducted based on three parameters: (i) ability to breed (ii) gestation period and (iii) clutch size.

CHAPTER 2

Gravid Spot Predicts Developmental Progress and Reproductive Output in a Livebearing Fish, *Gambusia holbrooki*

***This chapter has been published as follows:**

Norazmi-Lokman, N.H., Purser, G.J. and Patil, J.G. 2016. Gravid spot predicts developmental progress and reproductive output in a livebearing fish, *Gambusia holbrooki*. *PLoS One,* 11**,** e0147711.

2.1. Abstract

In most livebearing fish, the gravid spot is an excellent marker to identify brooding females, however its use to predict progress of embryonic development, brood size, timing of parturition and overall reproductive potential of populations remain unexplored. Therefore, to understand these relationships, this study quantified visual attributes (intensity and size) of the gravid spot in relation to key internal development in *Gambusia holbrooki*. Observations show that the colour of the gravid spot arises from progressive melanisation on the surface of the ovarian sac at its hind margin, rather than melanisation of the developing embryos or the skin of the brooding mother. More importantly, the gravid spot intensity and size were closely linked with both developmental stages and clutch size, suggesting their reliable use as external surrogates of key internal developmental in the species. Using predictive consistency of the gravid spot, we also determined the effect of rearing temperature (23°C and 25°C) on gestation period and parturition behaviour. The results show that gestation period was significantly reduced ($F=364.58$; $df=1,48$; $P<0.05$) at 25°C. However there was no significant difference in average number of fry parturated in the two temperature groups ($P>0.05$), reaffirming that gravid spot intensity is a reliable predictor of reproductive output. The parturition in the species occurred predominantly in the morning and in contrast to earlier reports, tails of the fry emerged first with a few exceptions of head-first, twin and premature births. This study demonstrates utility of the gravid spot for downstream reproductive investigations in a live-bearing fish both in the field and laboratory. The reproducibility of the relationships (intensity with both developmental stage and clutch size), imply that they are also relevant to wild populations that experience varying temperature climes and stressors, significant deviations of which may serve as indicators of environmental health and climate variability.

Keywords: Gambusia, Invasive fish, Live bearing fish, Reproduction, Development.

2.2. Introduction

The eastern mosquitofish *Gambusia holbrooki* is one of the most widely introduced and invasive freshwater fish species in the world (Pyke, 2005). Closely related to the western mosquitofish *G. affinis* and often discussed together as Gambusia, the life history of both species is of interest due to their utility as models of viviparity, mosquito control, environmental pollution and of late as a threat to native aquatic biodiversity (Pyke, 2008, Rautenberg et al., 2015). Originally considered as worthless, Gambusia were regarded as effective and beneficial to control mosquito and hence translocated worldwide to control the spread of malaria (Howard et al., 2007, Tiwari and Gaur, 2007, Chandra et al., 2008) during the early to mid-20th century. However they are now known as a pest and harmful to the native species in places of their introduction (Goodsell and Kats, 1999, Ivantsoff and Aarn, 1999, Hamer et al., 2002, Ling, 2004, Morgan et al., 2004, Keller and Brown, 2008, Rowe et al., 2008, Macdonald et al., 2012), needing urgent solutions for control (Patil, 2012). Predominantly, most available biological information is restricted to *G. affinis* with a general assumption that *G. holbrooki* also has a similar biology and behaviour, often leading to confusion and incorrect inferences. For example, there are significant differences in terms of morphology, chromosome structure and genetic makeup (Pyke, 2005), yet shared gamity (WZ/ZZ) between *G. holbrooki* and *G. affinis* based on information of the latter has been assumed even in recent times (Thresher et al., 2013). Systematic investigations therefore are necessary to resolve species-specific differences that are likely to yield novel insights into basic vertebrate biology, such as reproductive strategies and evolution as well as application of this knowledge to environmental pollution and management of invasive populations among others.

The detailed embryonic life stages of *G. affinis* have been described (Chambolle et al., 1970, Koya et al., 2000) but not for *G. holbrooki*. As in other viviparous teleosts, fertilization in *G. holbrooki* occurs internally where fully matured oocytes are fertilized within the ovary by spermatozoa held following copulation in an intra-ovarian structure known as a 'delle' (Jobling, 1995, Koya et al., 2000, Bone and Moore, 2007). The embryos develop in the ovarian follicles during the gestation period and are born as juveniles (Bone and Moore, 2007) with fertilization of the next clutch thought to occur after the birth of the previous clutch (Koya et al., 2000). Critically, the staging of embryonic development in the species necessitates sacrifice of brooding mothers precluding multiple time point observations (i.e. pre-and post-parturition). Most livebearers, including *G. holbrooki* also exhibit asynchronous embryonic development and parturition with overlapping and variable clutch size - often limiting an ability to predict reproductive output in wild fish or conduct experiments such as sex reversal in the laboratory.

Some Gambusia species also possesses an anal spot (a dark spot along the ventral midline close to the anal pore) that has been used as a tool to predict their reproductive cycle. Several studies on the life history of Gambusia have suggested that the size and pigment intensity of an anal spot can be linked to the female's reproductive cycle — the spot becoming larger (Peden, 1973a) and intensely pigmented (Pyke, 2005, Edwards and Guillette, 2007, Agrillo et al., 2008) with advancing maturity. However, no study has reported the occurrence of an anal spot in *G. holbrooki* and our observation on the introduced Tasmanian population suggests that they do not possess the anal spot, suggesting the need for an alternative external surrogate to predict the reproductive status of a pregnant female

Like most poecilid fishes, one of the distinct features that differentiate *G. holbrooki* females and males is the dark pigmented spot known as the gravid-spot (lateral and cranial to the anal/genital pore) possessed by mature females. While the gravid spot is an excellent marker to identify mature and brooding females its function and relationship to the *G. holbrooki* reproductive cycle has not been fully explored. As a first step towards understanding the origin and function of the gravid spot and to address limitations associated with developmental and reproductive investigations in this viviparous fish, we tested if the size and pigment intensity of the gravid spot could serve as an external and non-invasive indicator for developmental staging as well as to predict the timing of parturition and clutch size. Two different experiments were conducted in this study to achieve the objectives of: i) relationship between gravid spot and developmental progress; and ii) observation on gestation and parturition in this species. They were conducted sequentially where the results obtained in the first experiment were used to structure the second experiment, with the two rearing temperature regimes testing robustness/plasticity of the relationships.

2.3. Materials and methods

2.3.1. Source of specimens

Wild fish collection, handling and transportation were carried out as stipulated under the Inland Fisheries Service Tasmania permit and where necessary fish were euthanased using benzocaine. All procedures were also reviewed and approved by the University of Tasmania Animal Ethics Committee (AEC A12787). Stocks of *Gambusia holbrooki* used in this experiment were collected using dip nets in September 2013 from the Tamar Island Wetland Reserve (TWIR), Launceston, Tasmania. They were maintained in a recirculating aquaculture system (temperature: ±25°C; salinity: 0ppt; 16L:8D) at the Institute for Marine and Antarctic Studies (IMAS) Aquaculture Centre, University of Tasmania, Newnham, Tasmania. The fish were fed twice daily to satiation with commercial fish pellets (TetraMin® tropical granules, Germany).

2.3.2. Developmental staging

One hundred females displaying a range of sizes (20.0 – 50.0 mm SL) and maturity levels were chosen randomly, using the anal fin morphology as a guide to distinguish them from males. Prior to digital photography, fish were euthanased by an overdose of benzocaine (1:2000). The fish were then dissected and the eggs/embryos counted, measured and staged. In the absence of detailed developmental staging in *G. holbrooki*, a set of morphological staging criteria as described for *G. affinis* (Chambolle et al., 1970), was adopted for this study and broadly categorised into five stages (I-V) based on the morphological criteria (Table 2.1). In cases where there was more than one distinct embryonic stage in a female, the stage with the highest number of embryos was assigned to the female for the purpose of analysing the relationship between

gravid spot and developmental stages. Fecundity was measured as the total number of number eggs/embryos in each female (unfertilized eggs + embryos in each female) while clutch size was measured as the total number of embryos only.

Table 2.1. Criteria used for staging *G. holbrooki* embryos.

Developmental criteria*	Stage
Preliminary development of optic cupules/eyes.	I
Anterior neural tube broadened.	II
Eye pigmentation appears. Vitteline veins are visible.	III
Fine capillaries are developed on the surface of the pericardial sac. Appearance of melanophores on the tail. Gill slits are visible.	IV
Late developmental stage. First rays of the caudal fin become apparent. The mouth and nostrils are visible through the pericardial sac.	V

*Staging adopted from those described for *G. affinis* (Chambolle et al., 1970).

2.3.3. Digital imaging of the gravid spot

Photographs of the fish were taken under natural white colour bulbs on a white surface, using a DSLR camera (Nikon D3000 equipped with 18-55mm f/3.5-5.6G VR DX AF-S Nikkor lens, Japan). The camera was set at a fixed distance of 25 cm above the sample using a tripod. A colour reference card and a ruler were included in each photograph to allow comparisons between different photographs/individuals. All images were saved in RAW format for visual analysis (Yasir and Qin, 2009, Svensson and Sköld, 2011). Length of the fish, its gravid spot size (area) and spot intensity were measured using ImageJ software (Collins, 2007) in each image.

The intensity values are represented within a range of 0 (minimum value, black) to 255 (maximum value, white) (Stevens et al., 2007).

2.3.4. Gestation and parturition

The gestation period was observed in fish exposed to one of two temperatures: 23±1 and 25±1°C. Temperature was maintained by using room air temperature controllers and was recorded daily. Fifty pregnant females were chosen based on the gravid spot intensity value (intensity range: 28-38; values established from the first experiment) and randomly divided into two groups and exposed to the two temperature regimes. Each fish was held in individual static tanks (2.5L; 0ppt; 16L:8D) fitted with a breeding trap to prevent cannibalism by the mother. The brooding fish were fed to satiation twice daily with commercial fish pellets (TetraMin® tropical granules, Germany) and water changes were undertaken every two days to maintain water quality. Gestation period was measured as days between parturitions as exact timing of fertilization could not be ascertained.

Based on preliminary observations, parturition activity was closely monitored twice daily over a six month period: morning (0900-1100h) and evening (1500-1700h) and number of newborn fry were recorded at each time. The females were considered to cease reproducing if they did not show any signs of gestation such as swollen belly and when parturition did not occur for more than 50 days since the last parturition event. After parturition, the newborn fry were transferred to a separate rearing tank.

2.3.5. Statistical analysis

The data were analysed statistically by using IBM SPSS Statistic software (version 22). One-Way ANOVA was used to determine differences between the gravid spot intensity and the assigned developmental stages. The data was tested for normality and Pearson's correlation analysis was applied to determine the relationship between the following: the size of the gravid spot with the intensity, fecundity and total fish length; the intensity of the gravid spot with the fecundity and total fish length; and the fecundity and total fish length (Quinn and Keough, 2002). A multiple regression analysis to predict the clutch size with gravid spot size, its intensity and fish length as independent variables was also carried out.

2.4. Results

2.4.1. Gravid spot and embryonic development

Of the one hundred females examined, the presence of a gravid spot was observed in nearly all (92%) with intensity values ranging between 28-92. Developing embryos were found in 51 of these females while 39 possessed only mature eggs and 2 without any eggs. The total length of females without a gravid spot and eggs (n=8) ranged between 20.0-27.0 mm while the total length of females with a gravid spot ranged between 30.9-50.0 mm.

The gravid spot consisted of black pigments or melanophores that covered the ovarian sac. In females that possessed a gravid spot with a high intensity value (lighter/less dark), the eggs could be seen under a dissection microscope through the gravid spot (Fig. 2.1a). In females with embryos at the final developmental stage, the eyes of the embryos were visible to the naked eye despite the black pigmentation (Fig. 2.1b). Although the black pigment was scattered over the sac, it was most dense at the posterior margin of the embryonic sac, corresponding to the externally visible gravid spot (Fig. 2.1c). After the ovarian sac was removed from the body cavity, the area of the fish skin corresponding to the gravid spot appeared translucent (Fig. 2.1d).

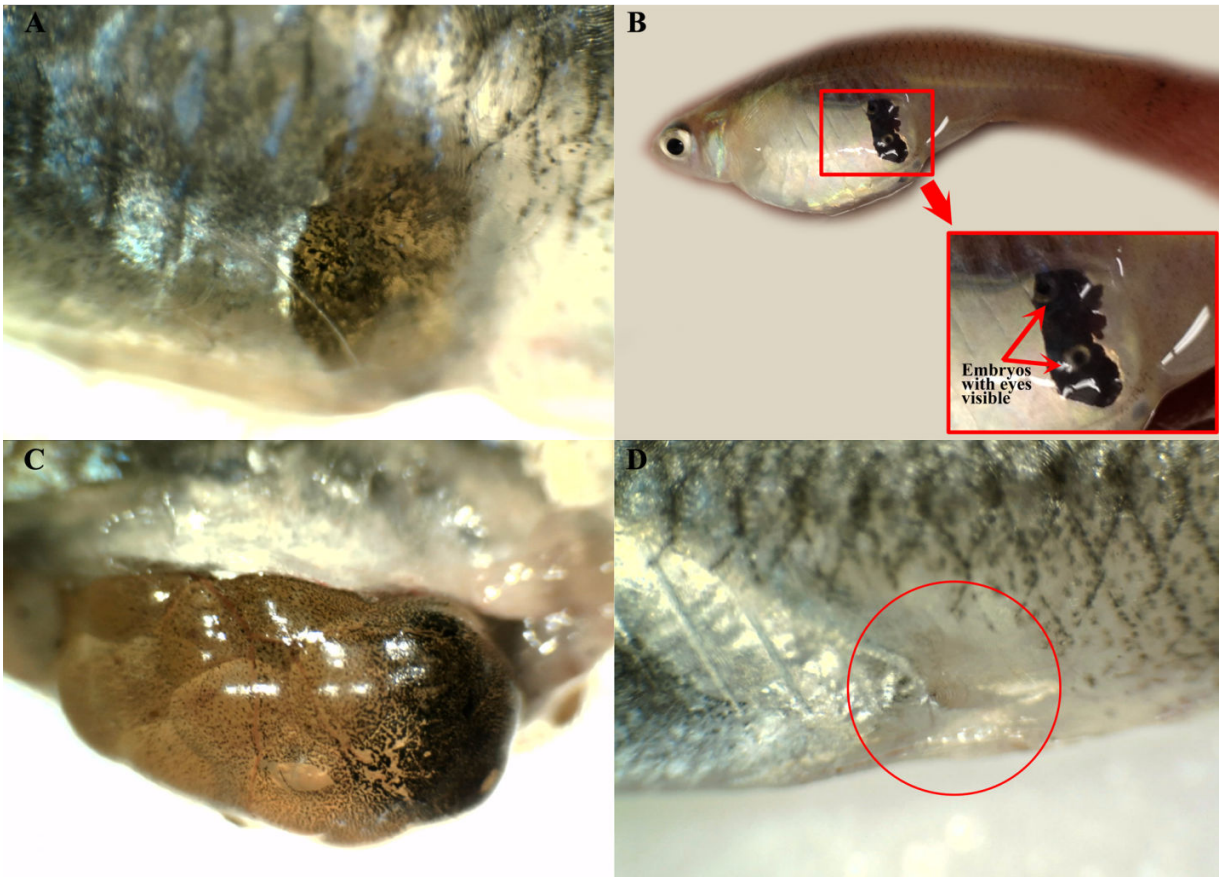


Fig. 2.1. Gravid spot of *G. holbrooki*. (A) Gravid spot of a female *G. holbrooki* with high intensity value (less dark). The yellowish colour of the yolk/eggs can be seen when observed under dissecting microscope. (B) Embryo's eyes at final developmental stage are visible through the gravid spot (red arrows). (C) Black pigment is scattered over the sac but is concentrated on the posterior margin of the ovarian sac. (D) Area of the skin corresponding to the gravid spot (circled red) appears as a translucent window after the ovarian sac was removed from the body cavity. Dorsal to the top and anterior to the left.

2.4.2. Embryonic Stages

Of the 92 females that possessed a gravid spot with eggs/embryo, 51 contained embryos or fertilized eggs. The number of embryos in each female examined ranged between 6 and 56 at different stages of development and fell into one of the five categories summarised in Table 2. Representative images including the unfertilised eggs are shown in Fig. 2.2.

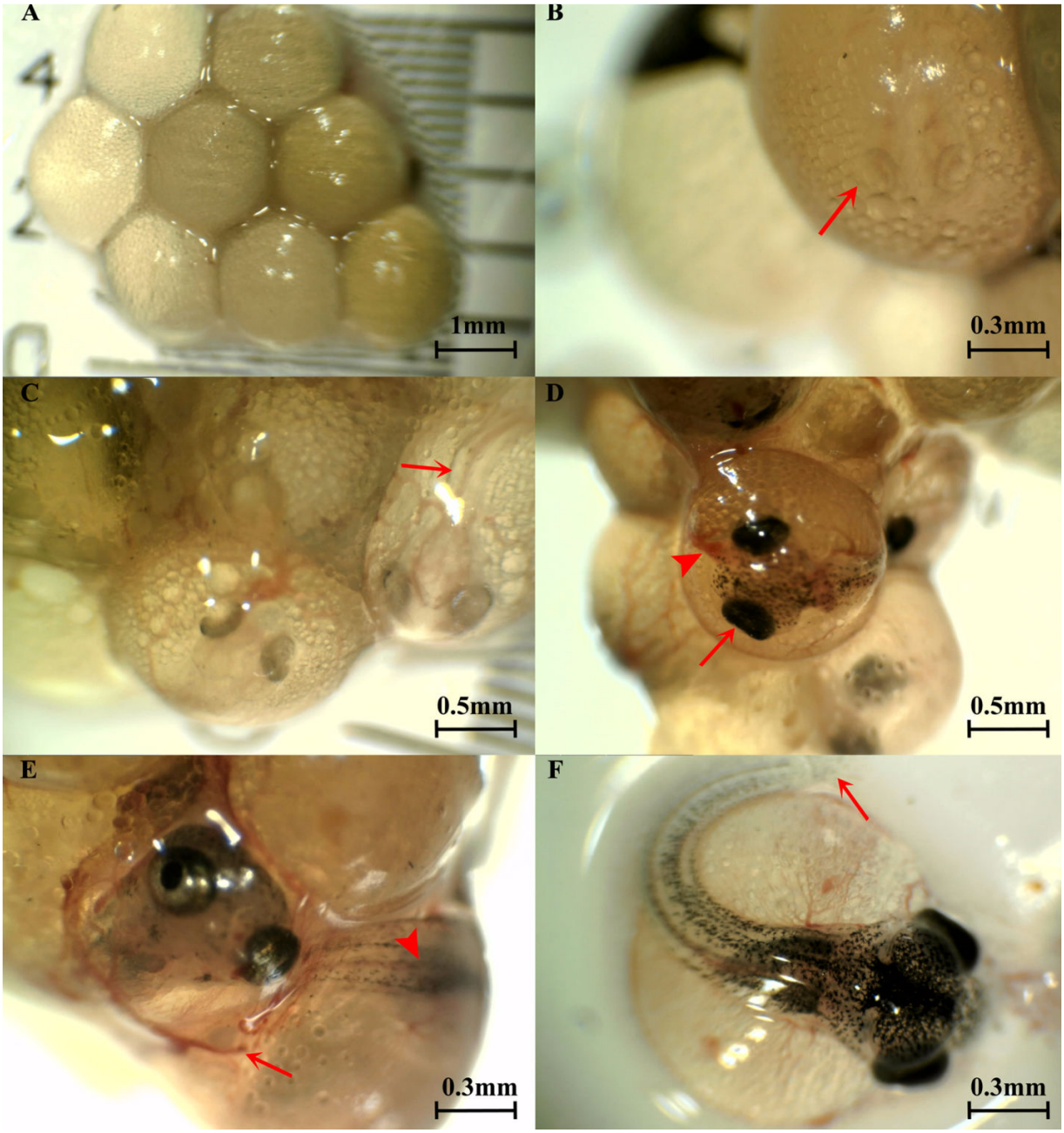


Fig. 2.2. Panel showing mature eggs and embryos of *G. holbrooki* at various developmental stages. (A) Mature unfertilized eggs with no sign of cell division; (B) Stage I embryos with rudimentary optic cupules/eyes (arrow); (C) Stage II embryo with anterior neural tube broadened (arrow); (D) Stage III embryo with eye pigmentation prominent (arrow) and visible vitteline veins (arrow head); (E) Stage IV embryo with fine capillaries on the surface of the pericardial sac (arrow), melanophores on the tail (arrow head) and visible gill slits and (F) Embryo at late developmental stage (Stage V), showing the caudal fin rays (arrow). At this stage the mouth and nostrils are also visible through the pericardial sac (Chambolle et al., 1970).

The clutches of embryos from 43 (84.3%) females were at more than one developmental stage (Figure 2.3 and Table 2.2). Invariably, all females with embryos also contained mature unfertilised eggs. The number of females with their assigned developmental stage is presented in Table 2.2.

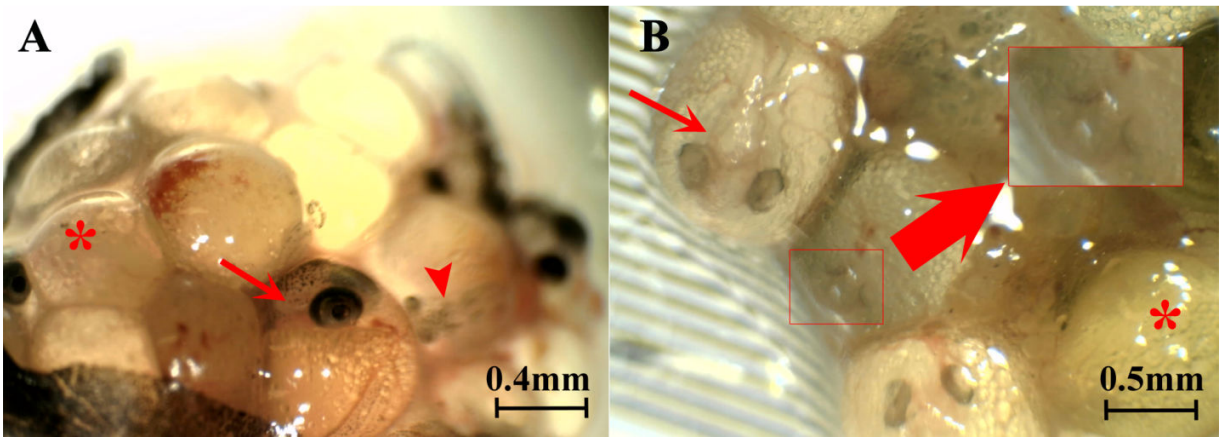


Fig. 2.3. Dissected ovarian sac of two *G. holbrooki* females showing embryos at multiple stages of development suggesting superfetation. (A). A developed (stage IV; arrow) as well as an early stage (stage II; arrow head) embryo alongside mature unfertilized eggs (asterisk). (B) Stage II (arrow) and stage I embryos (inset) together with mature unfertilized eggs (asterisk).

Table 2.2. Number of *G. holbrooki* females with overlapping developmental stages

Stages	Stage I	Stage II	Stage III	Stage IV	Stage V
Stage I					
Stage II	6				
Stage III	14	1			
Stage IV	9	1	-		
Stage V	8	2	2	-	

Note, no fish carried embryos with more than two developmental stages, but all the fish had unfertilized egg"

2.4.3. Relationship of gravid spot intensity and size with embryonic stage and fish length

The gravid spot intensity values observed in this study ranged between 28 (minimum) and 92 (maximum). Fig. 2.4 illustrates the range of gravid spot intensity values associated with the observed embryonic developmental stages. One-way ANOVA analysis confirms that there is a significant difference ($F=127.42$; df: 4, 47; $P<0.05$) between the intensity values corresponding to each embryonic stages of development.

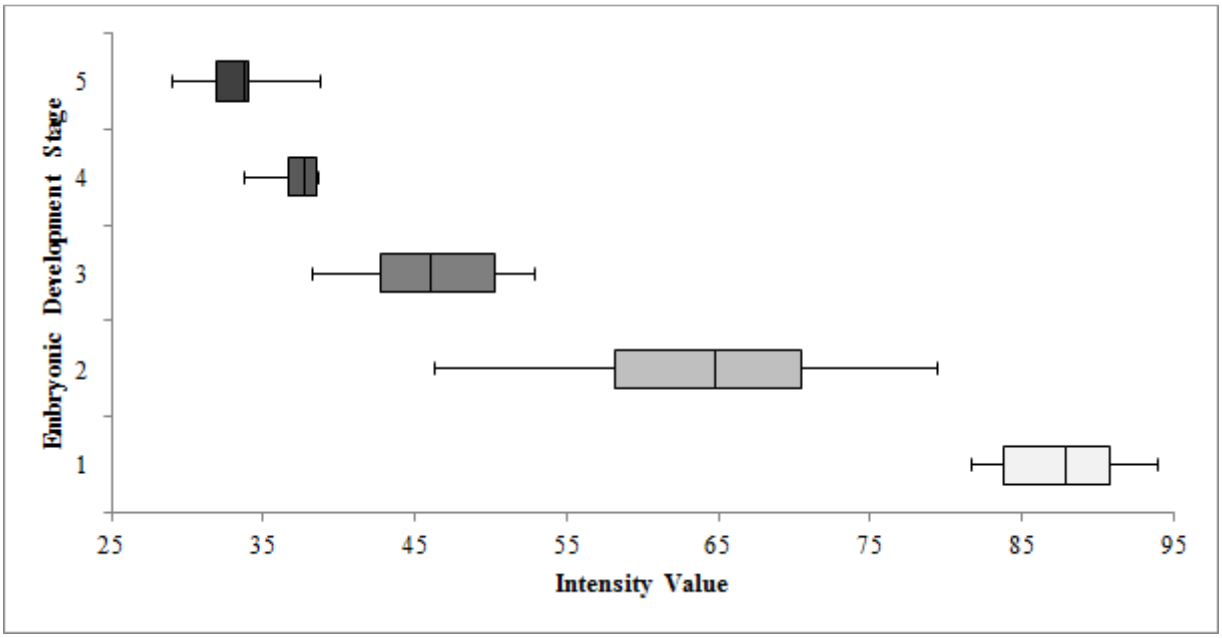


Fig. 2.4. Range of gravid spot intensity values corresponding to fish with assigned embryonic stages of development. As fish carried more than one developmental stage (generally 2) they assigned to the most dominant developmental stage they carried.

Pearson's correlation analysis showed a significant relationship between the intensity of the gravid spot with the size of the females (r=-0.60, n=92, P<0.01; Fig. 2.5a) and fecundity (r=-0.54, n=92, P<0.01; Fig. 2.5b). The relationship between the fecundity and the size of the fish was also strong (r=0.66, n=100, P<0.01; Fig. 2.5c). The sizes of the gravid spot increased with the length of the fish (r=0.572, n=92, P<0.01; Fig. 2.5d), its intensity (r=-0.66, n=92, P<0.01; Fig. 2.5e) and fecundity (r=0.48, n=92, P<0.01; Fig. 2.5f). In the multiple regression analysis, the assumptions of linearity, independence of error, homoscedasticity, unusual point and normality of residuals were met. These variables (spot size, its intensity, and fish length) collectively and reliably (F=12.659; df: 3, 47; P<0.05) predicted the clutch size (CS):

$CS = 1.835 - (0.85\ X\ SS)\ +\ (0.196\ X\ SI)\ + (3.543\ X\ FL)$, where SS and SI are gravid spot size and intensity respectively, and FL is fish length. Regression coefficients and standard errors can be found in supplementary Table 1 (Appendix 2).

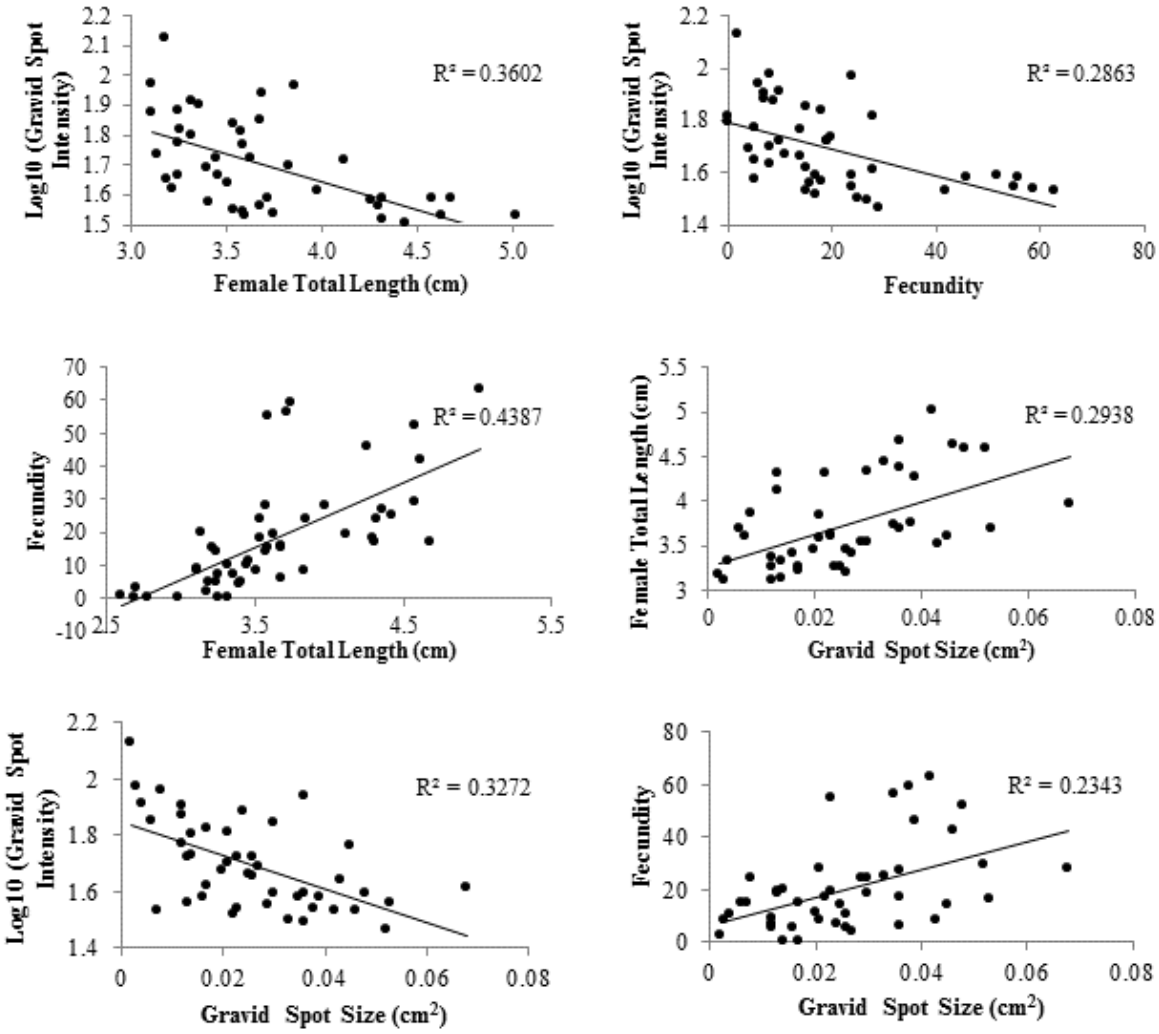


Fig. 2.5. Relationship between (A) gravid spot intensity and female size (total length); (B) gravid spot intensity and fecundity; (C) fecundity and female size (total length); (D) female size (total length) and gravid spot size; (E) gravid spot intensity and gravid spot size and (F) fecundity and gravid spot size. Note : Higher and lower intensity values represent lighter and darker coloration respectively.

2.4.4. Gestation period and parturition

The gestation periods of *G. holbrooki* females reared at two different temperatures are shown in Fig. 2.6. As expected, the average gestation period (number of days) for females reared at 23°C was significantly ($F=364.58$; $df=1,48$; $P>0.05$) longer (39±1.91 days) than those reared at 25°C (28.6±1.94 days), with clear range separation (Fig. 2.6) with an almost identical and uncanny range of 9 and 8 days between the quickest and the slowest gestation event at the 25°C and 23°C temperatures respectively.

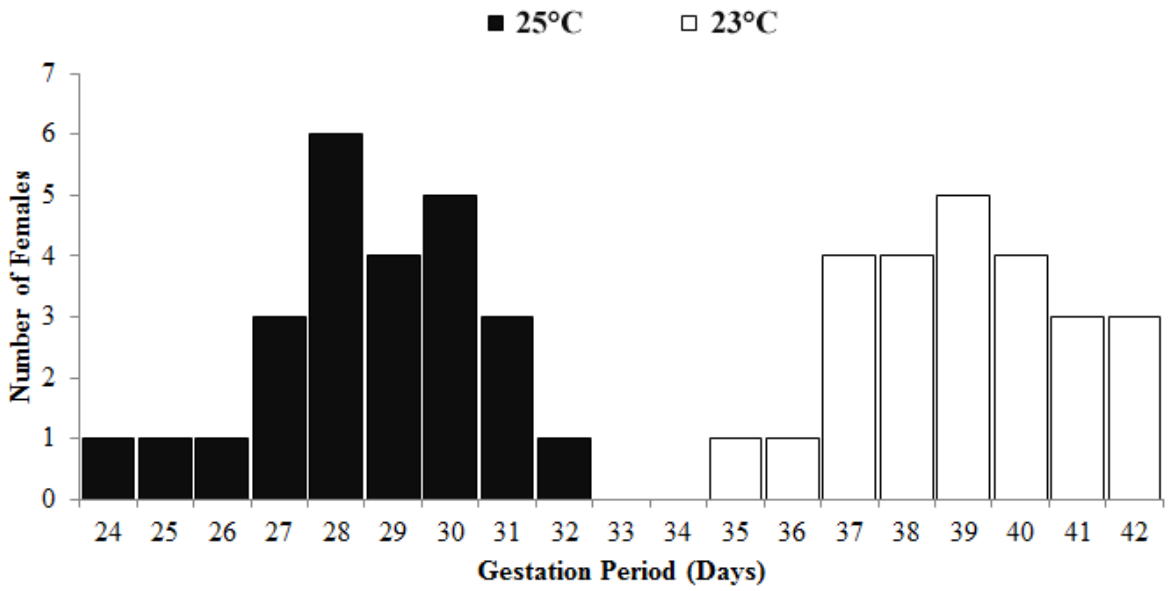


Fig. 2.6. Gestation period of *G. holbrooki* at two different rearing temperatures.

Based on observations, parturition in *G. holbrooki* occurred predominantly in the morning (0900-1100h) at both temperatures (Fig. 2.7a and 2.7b). At 23°C, 22 out of 25 (88%) females parturated in the morning while the number was 23 (92%) for females reared at 25 °C. The remaining females (3 and 2 at 23°C and 25°C respectively) parturated in the afternoon (1500-1700h). There were no significant differences ($P<0.05$) in timing (morning v/s evening) of birth between the rearing temperatures. Typically the duration of the parturition event ranged from 5 minutes up to 3hrs depending on clutch size, by which time all the fry in the clutch were delivered.

Three types of parturition postures were encountered. They were tail first; head first and twin births (see supplementary video - Appendix). The frequency of occurrence for each parturition posture is shown in Fig. 7a and b. Each of these events occurred randomly, for example, in one female, the first two fry were born tail first, whereas the third fry was born head first followed by tail first again for the 4^{th} - 11^{th} fry, followed by twins (2 fry released simultaneously). There were no differences ($P<0.05$) in the frequency of birthing postures between the two rearing temperatures.

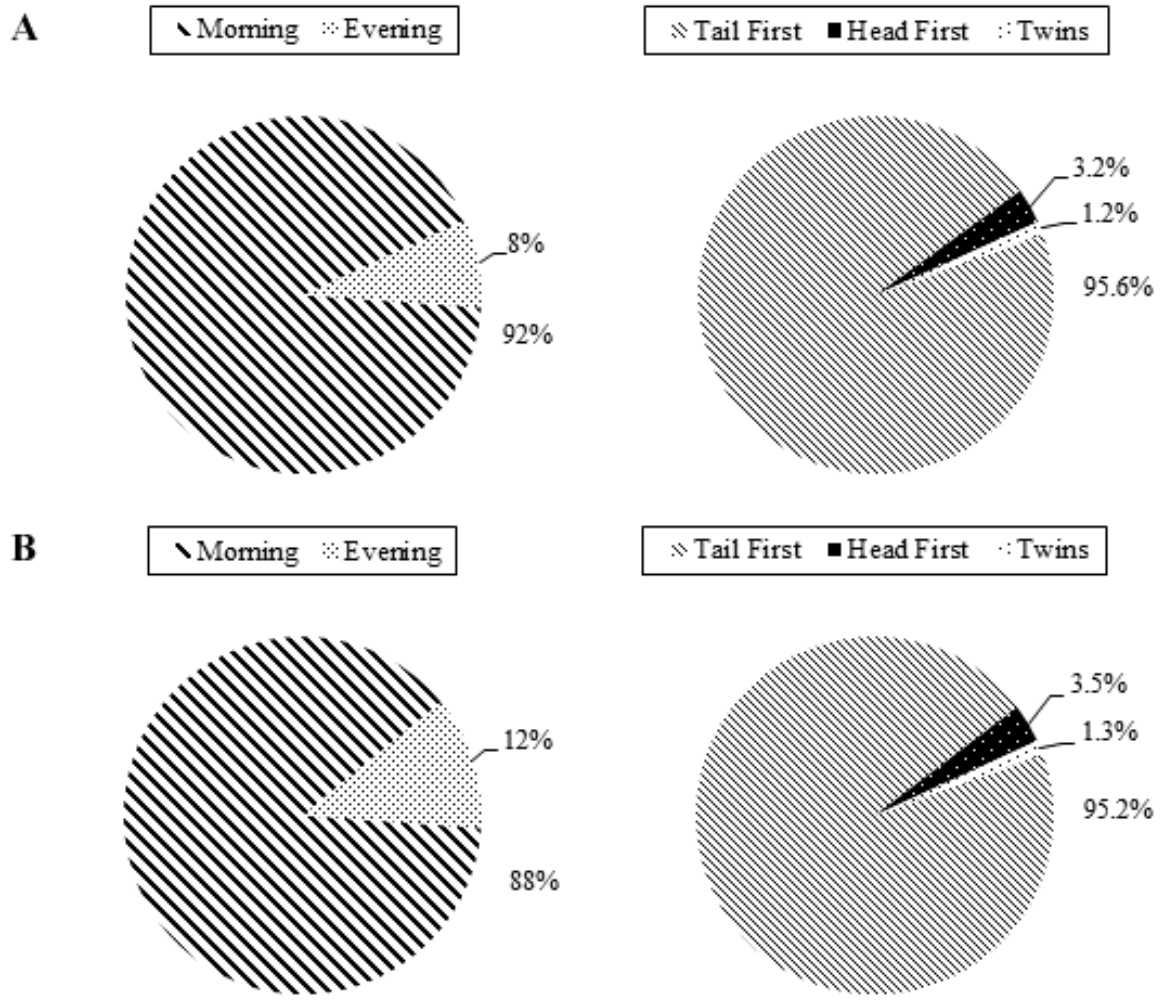


Fig. 2.7. Frequency of parturition timing (left) and postures (right) in *G. holbrooki* at: (A) 25°C and (B) 23°C.

Representative photographs of the birthing posture are also presented in Fig. 2.8. An event where the tail of newborn fry emerged first followed by one with a head-first is shown in Fig. 2.8a and 2.8b respectively. Typically, the curled fry, in the sac, positioned itself at the opening of the genital pore followed by an uncurling action of the tail, expelling itself (tail) out of the genital pore. The head and hence the newborn was later released between 15 to 40 seconds after the emergence of tail, usually preceded by a wiggle of the tail. A few seconds later, the tail of the second fry emerged and the process continued till the last fry in the clutch was born. In some cases, the tail of the next fry to be born emerged before the one ahead was completely released from the mother (Fig. 2.8b), with birth of two fry synchronised resulting in 'twin births' (Fig. 2.8c) on odd occasions.

Commonly, the yolk sac was completely absorbed at the time of birth. However, in fry from three females (coincidentally all reared at 25°C), the yolk sac was still intact at the time of birth (Fig. 2.8d). In these instances, the females showed signs of difficulty and stress during the parturition process as this lasted longer (5-10 minutes) than usual (15 to 40 seconds) and the survival of the babies was very poor, with only those delivered with yolk sac intact surviving (8e) while those where the yolk sac burst during the parturition event died instantly after birth.

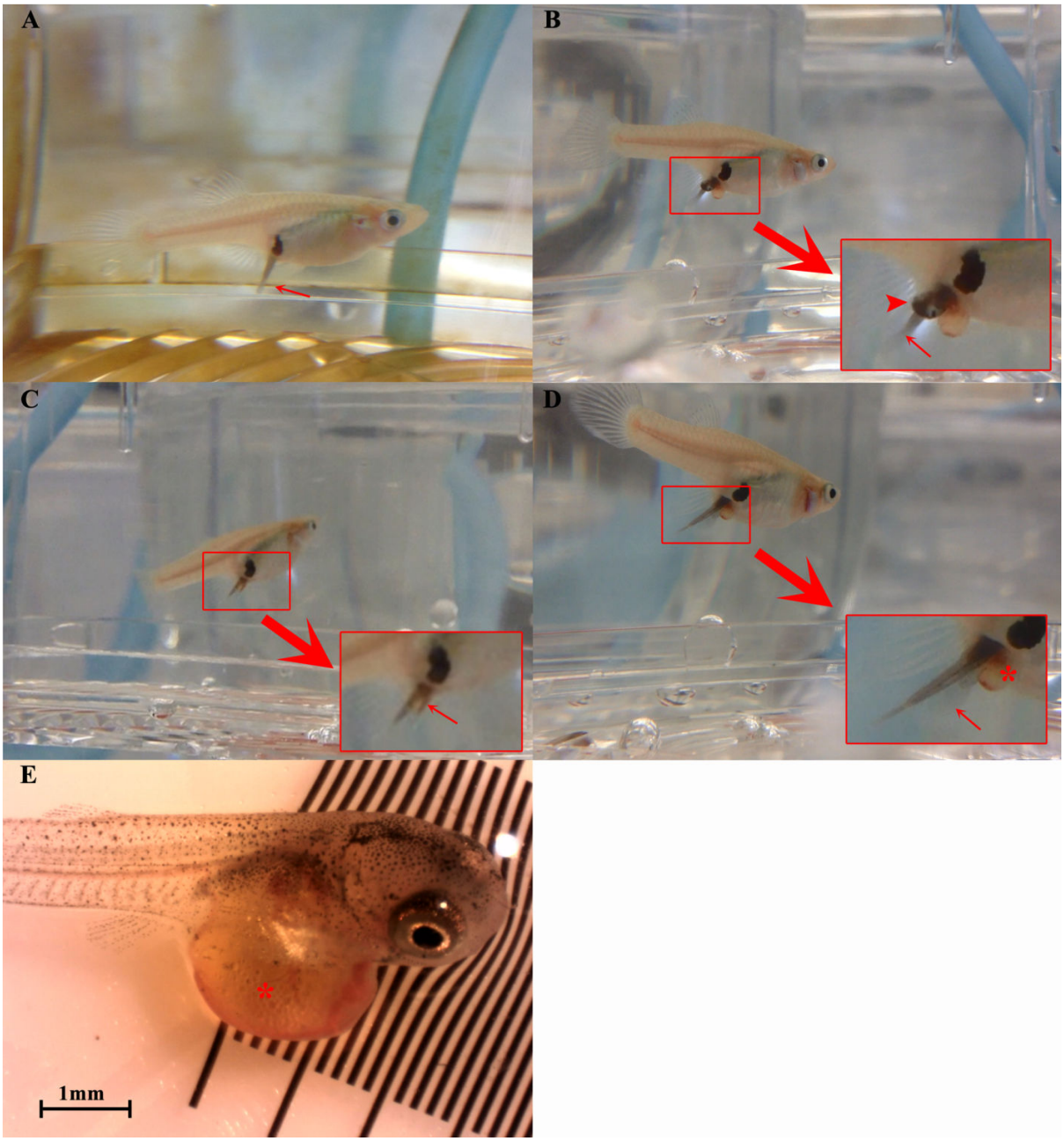


Fig. 8. Panel summarizing the parturition process in *G. holbrooki*. (A) Most frequently observed "tail-first" parturition event where tail of the fry (red arrow) emerged first from the genital pore; (B) Infrequent 'head-first' parturition event where the head (arrow head) of a fry emerged first from the genital pore. In this example, note the tail (red arrow) of the next fry born emerged before the one ahead was completely released; (C) On few occasions 'twin births' where two fries could be seen emerging simultaneously out of the genital pore (red arrow); (D) Parturition event showing a fry being born with yolk (asterisk) still intact. Note, the free tail (arrow) with head still stuck in the ovarian sac; (E) A safely born fry with the yolk sac (asterisk) still intact. Also see video (Supplementary video 1).

2.5. Discussion

The gravid spot has been known as an indicator of maturity in female livebearing fish such as Gambusia (Peden, 1973a, Howell et al., 1980) but its origin, function and relationship to the respective developmental and reproductive traits was previously not well-defined, particularly in *Gambusia holbrooki*. Discussed here are our observations on the source of gravid-spot pigmentation, its relationship to developmental progress and potential reproductive outputs in the species.

2.5.1. Source of gravid spot colour

This study shows that the gravid spot derives its colour from the black pigments covering the ovarian sac. This was particularly obvious when the area (of the fish skin) corresponding to the gravid spot became colourless/translucent as soon as the ovarian sac was removed from the brooding fish. These observations in part support the ideas of Peden (1973b) who suggested that the gravid spot is 'formed' by the tearing of peritoneum in the abdominal cavity underneath the fish skin, exposing the egg/embryo sac and its pigments and expanding in size with maturity.

Our observations show that gravid spot colour is physically derived from the hind margins of the embryo sac, where its dark pigmentation is most concentrated and intensifies as the embryos advance in development. In contrast, the gravid spot in those individuals with only unfertilised eggs was consistently pale. Taken together, the increased darkness of the spot with advancing development may imply that it is used to ward off or confuse the males, perhaps by way of mimicking a 'second' pair of eyes—protecting both the female and developing young from the

potential damages of gonopodial thrust of a mating male. It is well documented that the male poecilids including Gambusia possesses gonopodia with serrated tips, hooks and claws as accessory structures to facilitate coercive copulation (Greven, 2005, Langerhans, 2011, Kwan et al., 2013) and cause injuries/bleeding at the female genital pore (Rosen and Tucker, 1961). Conceivably, the fragile developing embryos are at greater risk of damage should copulation occur during 'pregnancy'/gravidity. Observation that male Gambusia avoid mating with females closer to parturition (Deaton, 2008) also support the notion that the gravid spot serves the purpose of repelling/warding off mating males during the period of gestation -more so closer to parturition, when the developing embryos are most vulnerable.

It is as yet unknown what mechanism/s triggers the progression of melanisation on the egg sac but it is likely linked to physiological responses in sync with elevated steroid hormone levels. For example, several studies have suggested that elevated steroid hormone levels regulate nuptial coloration in fish species such as the medaka (*Oryzias latipes*), guppy (*Poecilia reticulata*) and two-spotted goby (*Gobiusculus flavescenes*) (Niwa, 1965a, Niwa, 1965b, Toft and Baatrup, 2001, Toft and Baatrup, 2003, Sköld et al., 2008) as melanophores exhibit higher motility compared to other types of chromatophores when stimulated by nervous or hormone activity i.e. triggering aggregation of melanophores (Fujii, 1993). Nevertheless, further studies are needed to confirm and identify the hormones that might cause the migration, concentration and dispersal of melanophores that regulate the gravid spot colour intensity, during and after gravidity in *G. holbrooki* and poecilids in general. Noting that most previous studies (Niwa, 1965a, Niwa, 1965b, Toft and Baatrup, 2001, Toft and Baatrup, 2003, Sköld et al., 2008) have focused on

pigments located externally on the fish skin, this study should help direct future investigations to internal pigmentation of the embryonic sac.

2.5.2. Gravid spot can predict developmental progress, clutch size and timing of parturition

As has been demonstrated by this study, the gravid spot in *G. holbrooki* can be used to predict the embryonic development, clutch size and timing of parturition in females. Interestingly, the gravid spot appears semi-autonomous in that it does not physically derive its coloration from the developing embryos, but yet displays remarkable association with the progression of development and reproductive output. This established relationship should simplify future studies, both in the field and laboratory particularly in instances where the use of the gravid spot as an external marker could reduce/avoid the need to sacrifice brooding females and facilitate close synchronisation of developmental and reproductive status between individuals as may be critical in most comparative experiments.

Our observation shows that the size and shape of the gravid spot between individuals varies. For example, a smaller gravid female might possess a larger spot compared to a bigger female that may be undergoing recrudescence. This was also quite obvious from observations post-parturition, where the gravid spot decreased in size initially before regaining in size as the female goes through its next reproductive cycle. This is in agreement with observations of Howell et al. (1980) in this species where the size of the gravid spot increased with progression of gestation, albeit shrinking and expanding between successive gestation cycles.

As suggested previously (Peden, 1973b) the gravid spot is 'formed' or more accurately revealed due to the tearing of the peritoneum, its actual shape and size in each individual may be determined by how the peritoneum is 'ruptured'. Therefore like a fingerprint, the pattern of the gravid spot appears unique to each individual, a feature potentially useful in certain studies for example, in behavioural studies where the identification of individuals without intrusive external marking is important. However, it must be noted that both size and shape of the gravid spot display plasticity associated with swell and shrink of the belly associated with gravidity and recrudescence, respectively.

To our knowledge, this is the first study that quantifies and demonstrates a direct relationship between the intensity and size of the gravid spot, with key developmental and reproductive traits in a livebearing fish. This in part was facilitated by recent advances in digital imaging and analysis (Nordeide, 2002, Stevens et al., 2007, Yasir and Qin, 2009, Kouba et al., 2013). With the development of software such as 'ImageJ' (Collins, 2007) and 'Expertomica Fishgui' (Urban et al., 2012), the process of analysing digital images especially in terms of colour intensities has become easier, faster, much more accurate and are also increasingly amenable to automation. In contrast, studies on Gambusia's anal spot intensity and size (Hubbs, 1959, Peden, 1973a) that were carried out long ago were done by visual scoring making them prone to subjective error.

The utility of the gravid spot intensity in informing and refining developmental and reproductive studies was immediately apparent from the gestation and parturition experiments conducted as part of this study. Here the selection of the females of comparable reproductive status was facilitated by the intensity of gravid spot. Only females with a gravid spot intensity category of V

were chosen and all females parturated within 1- 5 days post-transfer to the individual tank providing greater control over design of downstream experiments and their observation in a timely and more organised fashion. In contrast, when chosen randomly we routinely encountered differences in timing of parturition in excess of 10 days between females - delaying timely inferences, prolonged wait between experiments and needless to say extra infrastructure costs including animal rearing and labour associated with the observations. The greater certainty offered by the intensity values in predicting the developmental progress and timing of parturition would assist in improving accuracy in developmental studies. Similarly, improvements in accuracy in pollution exposure studies are also easily conceivable. For example, repeat measure analysis associated with exposure to endocrine disruptors could potentially be quantified more readily, reliably, easily and potentially non-invasively, without the need for sacrificing the animals.

2.5.3. *G. holbrooki* exhibits superfetation during gestation

Scrimshaw (1944) reported that almost all poecilid species he examined, including *G. holbrooki* exhibit occasional superfetation i.e. the presence of more than one clutch of developing embryos in the same female at the same time, and suggested that it is possibly just a variation within a litter and does not mean that the embryos are from a different litter. It was also suggested that the embryos in the earlier developmental stage are underdeveloped and will be reabsorbed. Several other earlier studies have reported that in the genus Gambusia, superfetation does not commonly occur (Scrimshaw, 1944, Scrimshaw, 1945, Reznick and Miles, 1989, Reznick et al., 1996). In the current study, the observation of embryos at more than one development stage together with mature unfertilized eggs in most brooding mothers, suggests that superfetation is a norm and not

an exception in this species. Furthermore, in most females the differences between the embryonic stages were quite significant and tended to fall into two distinct stages. For example, in one female, that contained 24 embryos, 19 of them were at stage II while the remaining five were significantly advanced (stage V) and ready for parturition.

Interestingly, observations on the embryonic development in females of wild *G. holbrooki* populations in Tasmania (Keane and Neira, 2004) did not find any sign of superfetation in this species. Scrimshaw (1944) has suggested that superfetation is uncommon in most poecilids but that it can occur under favourable natural environmental conditions or under special laboratory conditions of constant lighting and unlimited food supply. For example, the occurrence of superfetation in least killifish (*Heterandria formosa*) was high during spring and summer but low during autumn and winter while superfetation was high in guppies (*Poecilia reticulata*) exposed to constant artificial light under laboratory conditions. In molly (*Poecilia sphenops*), superfetation is reported to occur only under very favourable environmental conditions and not under captive conditions in the laboratory (Scrimshaw, 1944). On balance, it is likely that this species and perhaps most poecilids adopt a strategy of superfetation to optimise recruitment output when environmental conditions are favourable and default to single clutch gestation, when conditions are less optimal. It is however intriguing as to how they modulate/orchestrate such complex reproductive strategies and respond, for example, to changing environmental climes.

2.5.4. Gestation is shortened by increased rearing temperature

The current observation shows that temperature has a significant ($P<0.05$) effect on the gestation period of this species, where a slight increase in temperature (2ºC) reduced the gestation period by 26.67%. Conceivably, an increased temperature significantly increased the rate of development resulting in reduced gestation period. The rate of embryonic development in fish in general is known to be significantly influenced by temperature—accelerated with increasing temperature within an optimal range, but retarded as it reaches the upper lethal threshold of any given species (For review see Rombough (1997)). The observed disproportional (> twice every 10 ºC) increase in gestation period at 23ºC suggests that the temperature is outside the optimal breeding range for the species and concur with an earlier suggestion that optimum breeding temperature for this species is 25-30°C (Keane and Neira, 2004). The observed average gestation periods (28.9 and 39 days at 25 and 23°C respectively) are comparable to those reported for wild populations (34 days) in Tasmania [50] and concur with observations in the guppy that the gestation is longer (40-60 days) when reared at lower than optimal temperatures (20°C and 23°C) (Dzikowski et al., 2001).

2.5.5. *G. holbrooki* predominantly parturates in the morning, with babies emerging 'tail-first'

Diel-timing of parturition in livebearers is known to vary from one species to another. For example, in *G. affinis* it is known to occur during early morning (Senior, 2013) while in guppies it is reported to occur in the evening (Rosenthal, 1951). Our observations show that *G. holbrooki* commonly parturates in the morning, very similar to *G. affinis*. The diel mechanisms that trigger parturition in each species are somewhat unclear. However, it has been shown that the parturition

in *G. affinis* can be stimulated by a sudden decrease of water temperature (Ishii, 1963), suggesting that dropping water temperature associated with dawn could provide the trigger. Nonetheless, the constant rearing temperature in this study and those of Senior (2013), suggests another factor particularly lights might play a greater role in triggering parturition in both *G. holbrooki* and *G. affinis*.

To our knowledge this is the first study to describe the parturition process in *G. holbrooki*. Our observations suggest that 'tail-first' birth is the norm in the species, with 'head-first' births constituting breech in this species. Similar observations of both head- and tail-first births also occur in the guppy (*Poecilia reticulata*) except that the head-first birth was the most frequent (Rosenthal, 1951). Earlier studies on *G. affinis* have reported that the tail emerged first with no mention of other patterns (Rosenthal, 1951, Tiwari and Gaur, 2007). The contrasting birthing postures may reflect species-specific reproductive strategies, providing clues for habitat/adaptive diversity and evolutionary relationships among live-bearing fish.

The observation that yolk is completely absorbed at the time of parturition is in agreement with those reported in guppy (Constantz, 1989), suggesting that the embryos born with yolk sac still evident are 'premature'. A similar premature birth condition was observed in guppies and swordtails, where birth of immature embryos and ova alongside live and well-developed fry was attributed to non-functional superfetation (Rosenthal, 1955). Thus, superfetation may in part explain the occurrence of premature birth in a few *G. holbrooki* females observed in this study. However, this is unlikely as in at least three females the entire clutch of fry was born prematurely and survived except for a few that were born with burst yolk sac. Reports in mollies and guppies

have attributed handling stress as the main reasons for premature births (Emmens, 2013, Arthur, 2013). It is more likely, the process of transfer of brood fish from stock to breeding tanks may have caused stress in the three pregnant females causing them to give birth prematurely.

2.6. Conclusions

This study demonstrates that the gravid spot can be used to predict the embryonic development, timing of parturition and clutch size of *G. holbrooki* facilitating better design and observation of downstream experiments. Predictably, the gestation period in this species is significantly influenced by temperature and parturition takes place mainly in the morning. The study also shows that the parturition in *G. holbrooki* occurs predominantly 'tail first' with few exceptions of 'head-first', twin and premature births. Future investigations on basic reproductive biology, including mechanisms of gravid spot melanisation, superfetation, sperm activation and triggers of parturition in this species as well as their utilisation in applied pollution research and management of pest populations will be greatly facilitated by the methods, observations and relationships established in this study.

CHAPTER 3

Paradoxical Effect and Gonadal Atrophy in the Eastern Mosquitofish *(Gambusia holbrooki)* Treated with Diethylstilbestrol

3.1. Abstract

Diethylstilbestrol (DES) is an endocrine disrupting compound (EDC) that due to its potency has been widely used in the aquaculture industry for the feminization of various fish species. This study investigated the efficacy of this synthetic estrogen in the feminization of the eastern mosquitofish (*Gambusia holbrooki*). Two parallel oral administration experiments were conducted: Experiment 1, indirectly treating the developing embryos via gravid females and; Experiment 2, direct treatment of newborn juveniles immediately following parturition. The concentrations tested in both experiments ranged between 20 to 100 mg/kg feed for a duration of 30 days. Two control groups were set for each experiment: (C1) normal feed (no chemical exposure) and (C2) feed mixed with 70% ethanol (vehicle control In Experiment 1, almost all females parturated however when examined after 30 days, ovarian atrophy was observed in treated females at all administered doses suggesting that DES precluded production of eggs in the subsequent reproductive cycle.. The mean survival rate (MSR) of the juveniles in the treatment groups was significantly lower ($F=16.79$; df: 6, 15; $P<0.05$) compared to the control groups (C1: 74.76±15.37%; C2: 71.26±18.5%) where the highest MSR was only 30.15±6.05 % in fish treated at the concentration of 40 mg/kg. Similarly, the MSR of treated juveniles in Experiment 2 was also significantly lower ($F=3.216$; df: 6, 28; $P<0.05$) than the control groups (C1: 78.42 ±22.0%; C2: 73.8 ±20.22%) with the highest MSR (45.6 ±17.93%) shown by fish treated with the lowest dose (20 mg/kg) of DES. There was no significant difference in the MSR between the treatment groups within both experiments. Chi-square analysis on gender ratios of control groups in both experiments showed that they were not significantly different from the expected 1:1 male:female ratio. In contrast, a paradoxical masculinization effect' was observed in all treatments of both experiments where the sex ratio was 100% male (phenotypically).

Observation of treated fish at 365 DAP revealed that they possessed a shorter under-developed gonopodium lacking serrated hooks compared to control and normal males. This is a first study reporting a paradoxical masculinization effect of DES in *G. holbrooki*. A paradoxical effect even at relatively low doses of treatment suggests that DES may not be a suitable feminising agent for *G. holbrooki* sex reversal. Nevertheless this study provides a diagnostic marker (i.e shortened and hook-less gonopodium) for detection and or predicting DES or DES like EDCs in the environment using *G. holbrooki* as an indicator organism.

Keywords: Gambusia, Invasive fish, Live bearing fish, Endocrine Disrupting Compound, Gonopodium.

3.2. Introduction

Diethylstilbestrol (DES), is a non-steroidal estrogen that was first synthesized in 1938 as an orally effective estrogen to be used for human medicinal purposes (Dodds et al., 1938). During early years, DES was prescribed to pregnant women to reduce the risk of miscarriage and other pregnancy complications (US Centre for Disease Control and Prevention, 2012, Wise et al., 2015) but it was later taken off the market when in 1971 it was found to cause seven types of rare cancer (Herbst and Scully, 1970, US Centre for Disease Control and Prevention, 2012). DES was also used in the livestock industry to stimulate growth and improve feed utilization in sheep and cattle (McMartin et al., 1978, Raun and Preston, 2002). The industry later discontinued the practice after the United States Food and Drug Administration (FDA) banned its usage in cattle production in 1979 due to the harmful effects of DES on humans.

Despite its harmful effects, DES has been widely used in the aquaculture industry for fish sex reversal due to its potency (Pandian and Sheela, 1995, Piferrer, 2001). Compared to cattle, steroid residues in fish disappear in less than a month after cessation of treatment (Piferrer, 2001) thus eliminating any risk if treated fish were used for human consumption. Diethylstilbestrol has almost the same potency as 17α-ethynylestradiol (EE2), on average two times more potent than estradiol (E2) and about eight times more potent than estrone (E1)(Piferrer, 2001) which makes DES much more favourable compared to other estrogens. The first successful usage of DES in fish feminisation was reported in medaka (*Oryzias latipes*) (Yamamoto, 1953) and since then DES has been successfully used to feminize other fish species such as the Nile and Mozambique tilapia (*Oreochromis niloticus* and *O. mossambicus*) (Tayamen and Shelton, 1978, Varadaraj,

1989), guppy *(Poecilia reticulata*) (Kavumpurath and Pandian, 1992) and black molly (*P. sphenops*) (George and Pandian, 1995).

Hormonal sex reversal both for feminization and masculinization has been achieved in other poecilids such as guppy, black molly and the western mosquitofish (*G. affinis*) (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b, George and Pandian, 1995, Senior, 2013). Poecilids are known to have three labile periods suitable for hormone treatment: during their embryonic development (treatment through gravid females), post-parturition and post-maturity (Pandian and Sheela, 1995, Pandian and Koteeswaran, 1999, Piferrer, 2001). Treatments during the latter two stages usually last between 7 – 50 days depending on hormone dosage and administration methods. Oral ingestion via feed as carriers is the most common method used in poecilids (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b, George and Pandian, 1995, Pandian and Sheela, 1995). Hormone administration by the immersion technique has been used in *G. affinis* but resulted in low survival rates despite successfully producing sex reversed individuals (Senior, 2013).

The livebearing eastern mosquitofish, *Gambusia holbrooki* is a noxious aquatic pest that was introduced to Australia in 1928 as a bio-control for mosquitoes (Rowe et al., 2008). Control and eradication efforts are currently undertaken to eradicate this species due to its harmful and threatening effect on native fauna. As manual and chemical approaches are ineffective a genetic approach of Trojan Sex Chromosome (TSC) proposed by Gutierrez and Teem (2006) has been proposed as an alternative solution to combat *G. holbrooki (Patil 2012)*. In fish species such as *G. holbrooki* with an XX-XY gamity (Angus, 1989a, Angus, 1989b, Horth, 2006), the TSC

involves the release to the wild of Trojan females with a YY chromosome configuration produced via hormone sex reversal (Gutierrez and Teem, 2006). However, there is no study that has conclusively demonstrated sex reversal in *G. holbrooki*, with the exception of a study published more than 50 years ago (Leproni, 1945) using oestrone (referenced in (Piferrer, 2001), the details of which are obscure. Most reports on sex reversal in *G. holbrooki* are restricted to the effects of environmental pollution i.e. endocrine disrupting chemicals (EDC) on juvenile and adult life stages (for example in Game et al. (2006), Brockmeier et al. (2013), Midgley et al. (2014)). Hence, detailed studies are necessary to determine the suitability of hormone, treatment dosage, route of administration and the appropriate life stage for treatment, so that precise and reliable sex reversal of *G. holbrooki* can be carried out on a routine basis. As a first step towards addressing these goals, this study investigated:

1. The effects of DES on the reproductive traits/outputs of the treated gravid females;
2. The effects of DES on the survival of treated embryos (via ingestion by gravid females) and newborn juveniles (direct feeding) and;
3. The efficacy of DES oral administration in feminizing *G. holbrooki*.

3.3. Materials and Methods

All procedures conducted in this thesis (Chapters two to five) have been reviewed and approved by the University of Tasmania Animal Ethics Committee (AEC A12787). The purchase and storage of hormones were undertaken under an approved permit issued by the Tasmanian state Department of Health and Human Service (Authority No.: R6/120926). This study was divided into two experiments: (i) DES treatment of gravid females and (ii) DES treatment on newborn juveniles. Both experiments were conducted concurrently. Reproductive biology and behaviour information obtained from Norazmi-Lokman et al. (2016)(chapter 2) were used to structure the sex reversal experiments.

3.3.1. Source of Specimens

Thirty-five gravid *G. holbrooki* females (size range: 31.0-46.0 mm TL) containing embryos at late developmental stages (gravid spot intensity value 28-38) (Norazmi-Lokman et al., 2016) were chosen from the stock tank (five fish per treatment including controls). The females were held individually in static tanks (2.5L, 0ppt, ±25°C; 16L : 8D – lights on at 06.00h) fitted with a breeding trap and left to parturate (Fig. 3.1A). All the tanks were supplied with gentle aeration. Female fish were fed to satiation twice daily (morning 0900-1000h; evening 1600-1700h) with commercial fish pellets (TetraMin® tropical granules, Germany). After the first parturition occurred (between 2-5 days after they were transferred into individual tanks), the juveniles were transferred into a static rearing tank (1.5L, 0ppt, ±25°C) that was supplied with gentle aeration (Fig. 3.1B) and were used in the second experiment while the females were retained for oral administration of the hormone (Experiment 1). Throughout the study, water quality of the rearing

tanks was maintained within acceptable limits by water exchanges which were undertaken every two days using aged and aerated tap water. Instead of using a recirculating aquaculture system (RAS), a static tank set up system was chosen to avoid cross contamination by DES especially of control tanks.

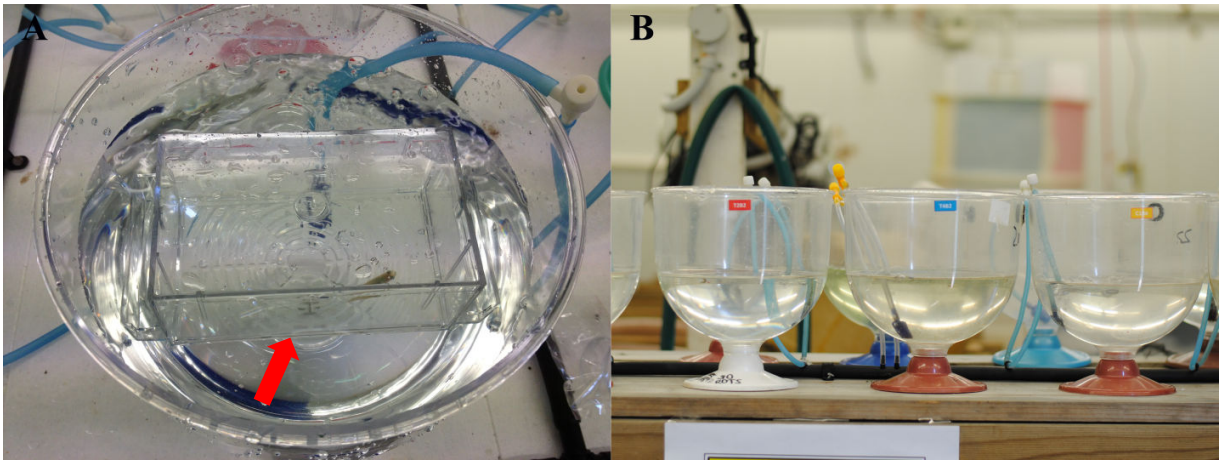


Fig. 3.1. Rearing tanks used for holding and treating fish in sex reversal experiments. (A) Gravid female was held in a static tank (Experiment 1) fitted with a breeding trap (arrow) and (B) newborn juveniles were held in 3L container filled with 1.5L water (Experiment 2).

3.3.2. Preparation of DES-enriched feed

Diethylstilbestrol (DES; CAS No.: 56-53-; Sigma-Aldrich, Germany) was administered orally in both experiments 1 and 2, via feed. Commercial fish pellets (TetraMin® tropical granules, Germany) were used to feed gravid females while the newborn juveniles were fed with DES-enriched powdered spirulina (Bioglan, Australia). Based on a preliminary experiment, five doses of DES were administered at 20, 40, 60, 80 and 100 mg/kg feed (labelled as T1 – T5) for both experiments. Two control groups were set for this study; Control 1 (C1): normal feed (no chemical exposure) and Control 2 (C2): feed mixed with 70% ethanol (vehicle control). Hormone stock solutions were first prepared by dissolving the required quantity of DES in 20ml

of 70% ethanol before it was mixed thoroughly with 20 g of the feed items. The mixture was then spread into thin layers on a tray and was allowed to dry in a fume hood for 24 hours at room temperature. The DES-enriched feed was then kept in a sealed container and stored at ±4°C (Phelps and Popma, 2000, Angus et al., 2005).

3.3.3. Experimental Design.

Both experiments were conducted concurrently. Fig. 3.2 illustrates the overall experimental design and workflow of both experiments.

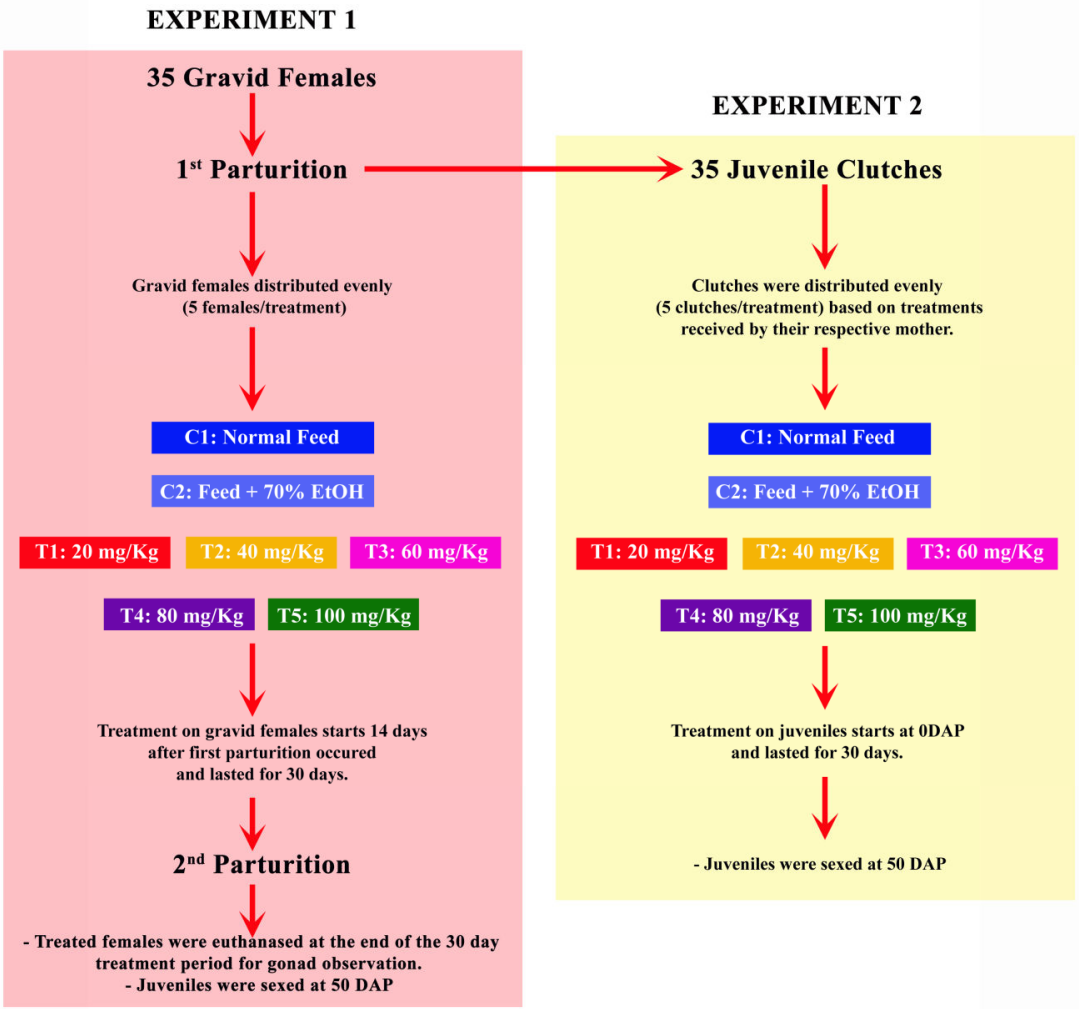


Fig. 3.2. Experimental design and workflow of the sex reversal experiments. DAP; days after parturition.

3.3.3.1. Experiment 1: DES treatment of gravid *G. holbrooki* females

The 35 gravid females each with a gravid spot intensity value between 28-38 (Norazmi-Lokman et al., 2016) were distributed randomly to the respective treatment and control groups (n=5 fish for each treatment group) and allowed to parturate. Following parturition the fry were transferred as a clutch to separate tanks for treatment, whilst the females were fed a control diet (for 14 days) allowing the females to prepare for the next parturition cycle (Norazmi-Lokman et al., 2016). After 14 days the adults were switched to respective hormone diets for 30 days. They were fed *ad libitum* (≈5% body weight) with respective DES-enriched diet twice daily. Progeny produced following treatment were transferred and reared in a static tank (1.5l, 0ppt, ±25°C; 16L : 8D) in separate groups. Gentle aeration was provided in each tank. The juveniles were fed with commercial powdered spirulina (Bioglan, Australia) for 30 days followed by weaning onto commercial micropellets (Aqua One, UK) over 10 days before the sex ratio was determined at 50 DAP by observing the morphology of the anal fin (Angus et al., 2005). The gestation period of the females, number of fry produced and survival rates of the progeny at 30 DAP, were observed and recorded. At the end of the treatment, all the treated females were euthanased by overdosing in benzocaine (1:2000) to facilitate examination of the morphology of the gonad. When no signs of maturity or breeding were observed, the individuals (progenies) were re-sexed 365 DAP.

3.3.3.2. Experiment 2: DES treatment of newborn *G. holbrooki*

The clutches of newborn juveniles (born before treatment of the mothers) were treated with respective DES enriched or control spirulina based on the concentration received by their mothers in Experiment 1. The treatment was initiated at 0 DAP and lasted for 30 days during which they were fed twice daily to satiation. After the treatment period, the juveniles were

weaned over 10 days onto commercial micropellets (Hikari®, Japan). The survival rates of the juveniles were recorded and they were sexed at 50 DAP using secondary sexual characters (Angus et al., 2005) and appearance of the gravid spot. As in Experiment 1, no sign of maturity or breeding was observed, therefore the fish were re-sexed 365 DAP.

3.3.4. Statistical Analysis

All data are presented as mean ± standard deviation unless otherwise stated. The data were tested for normality (Shapiro-Wilk normality test) and differences in gestation period and survival rates between the treatments were analysed using One-way ANOVA or Welch's ANOVA (for unequal sample size) followed by Tukey or Tukey Kramer (for unequal sample size) post-hoc test where applicable. Paired-samples t-test was used to analyse the differences between the number of progeny of the first and second parturition event (Quinn and Keough, 2002). Finally, the Chi-square (χ^2) test was used to analyse the sex ratio of the treatments and control groups. Differences were considered to be significant at $P<0.05$. All data were analysed using IBM SPSS Statistic software (version 22).

3.4. Results

3.4.1. Experiment 1: Gestation period, parturition and gonad morphology of *G. holbrooki* gravid female treated with DES

Of a total of 35 females treated in the experiment, 29 survived of which 22 parturated by the end of the treatment period. Seven females failed to parturate (two from C2, three from T1 and one each for T2 and T3) while six females from the two higher concentrations (four in T4 and two in T5) died before the end of the treatment period. The mean gestation period observed throughout the experimental period for the parturating females was 28.9±2.97 days with 24 days as the shortest period observed while the longest was 34 days. One-way ANOVA analysis shows that there were no significant difference in the gestation period between the treatments and controls. The exposure period of the embryos to DES (prior to parturition) ranged between 10 to 23 days. During the first parturition, the mean number of progeny produced was 12±8.4 while in the second parturition it was 7 ±4.7, a statistically significant decrease of 5 (95% CI, 2 to 9) progeny, ($t(23)=3.095$, $P<0.05$). Table 3.1 provides data on the number of progeny produced in each parturition event, gestation period and exposure period of embryo and females to DES.

Table 3.1. Number of progeny produced in each parturition event, gestation period and exposure period of embryo and females to DES.

Treatment	Female	1st Parturition* (#progeny)	Gestation Period (days)	2nd Parturition (#progeny)	Embryo exposure period (days)	Exposure Period (days)
C1 (without Ethanol)	F1	4	27	7	-	30
	F2	3	28	6	-	30
	F3	2	30	11	-	30
	F4	7	26	6	-	30
	F5	12	28	5	-	30
C2 (with 70% Ethanol)	F1	20	32	6	18	30
	F2	2	-	-	-	30+
	F3	4	30	3	16	30
	F4	5	34	14	20	30
	F5	7	-	-	-	30+
T1 20 mg/kg feed	F1	3	-	-	-	30+
	F2	8	-	-	-	30+
	F3	8	-	-	-	30+
	F4	19	26	4	12	30
	F5	21	28	16	14	30
T2 40 mg/kg feed	F1	7	25	3	11	30
	F2	7	-	-	-	30+
	F3	26	31	13	17	30
	F4	7	37	4	23	30
	F5	20	27	9	13	30
T3 60 mg/kg feed	F1	7	29	17	15	30
	F2	25	27	3	13	30
	F3	23	29	3	15	30
	F4	20	31	4	17	30
	F5	16	-	-	-	30+
T4 80 mg/kg feed	F1	1	-	-	-	22^
	F2	11	24	5	10	30
	F3	33	-	-	-	25^
	F4	18	-	-	-	23^
	F5	20	-	-	-	29^
T5 100 mg/kg feed	F1	14	-	-	-	25^
	F2	4	31	3	17	30
	F3	25	29	10	15	30
	F4	15	26	9	12	30
	F5	18	-	-	-	20^

*juveniles born prior to hormone treatment that were used in Experiment 2; ^ denotes individuals that died before the end of the treatment period; +second parturition did not occur.

At the time of terminating the experiment, the gonads of females in both control groups appeared to be normal (Fig 3.3). In a few females the gonad contained embryos at the third and fourth stage while some only contained unfertilized mature eggs (Fig. 3.3A). Compared to the controls, all females treated with DES (all concentrations) had shrunken gonads with enhanced dark pigmentation and only contained immature eggs (Fig. 3.3B). The gravid spot of the treated females also appeared to be smaller although there was little difference in the intensity compared to controls (Fig 3.3.C and D). Gonad content of controls and treated fish are summarised in Table 3.2.

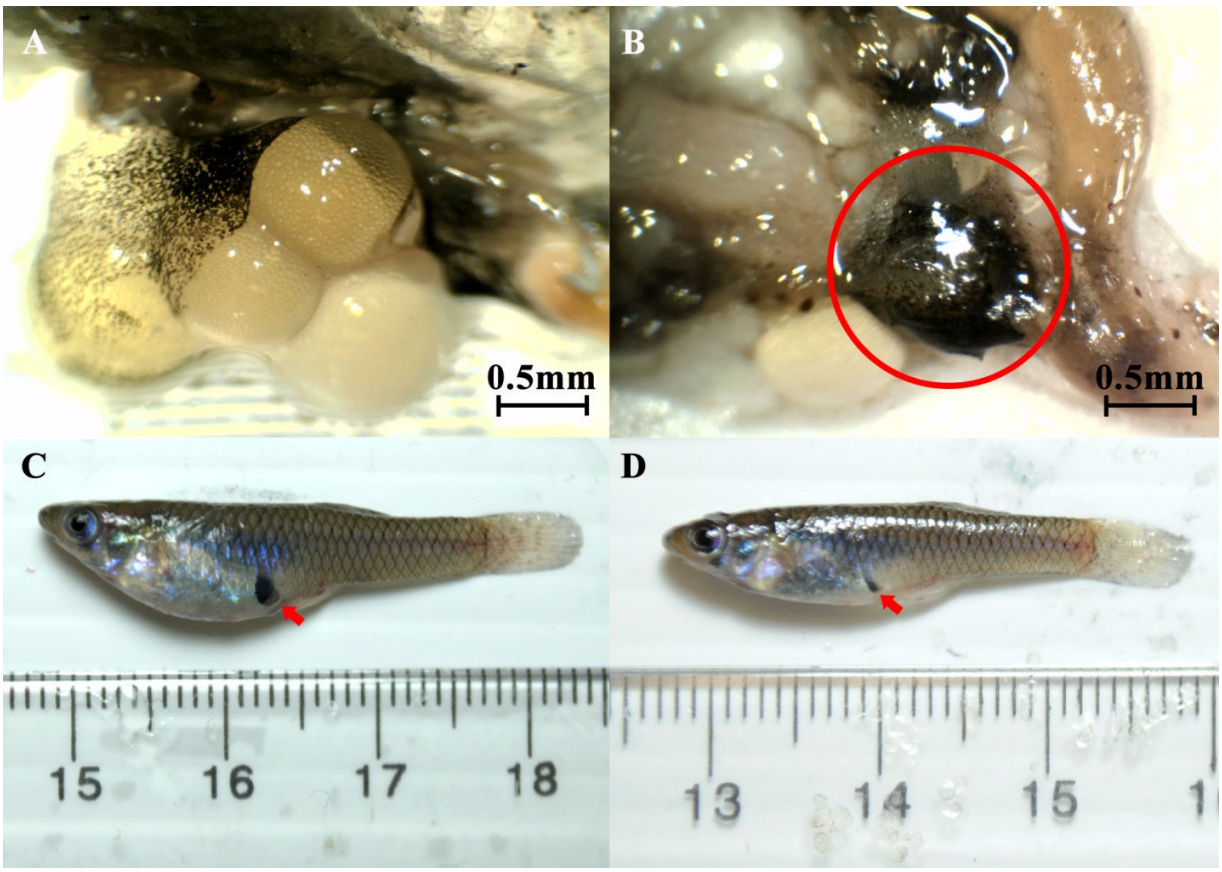


Fig. 3.3. Representative photograph of typical gross gonad morphology and gravid spot of *G. holbrooki* females at the end of the treatment period. (A) Gonad of control group female with unfertilized mature eggs. (B) Shrunken gonad of female treated with DES (circled red). (C) Gravid spot of control (C1; shown by red arrow) female (scale in cm). (D) Gravid spot of DES treated female (40 mg/kg feed; shown by red arrow) (scale in cm).

Table 3.2. Gonad content of controls and treated females at the end of DES treatment (30 days).

Treatment	Females	Gonad Content			
		Immature Eggs	Mature unfertilized Eggs	Third Stage Embryo	Fourth Stage Embryo
C1 (without Ethanol)	1		✓		
	2		✓	✓	✓
	3		✓		
	4		✓	✓	✓
	5		✓	✓	✓
C2 (with 70% Ethanol)	1		✓		
	2		✓	✓	✓
	3		✓		
	4		✓	✓	✓
	5		✓		
T1 - 20 mg/kg feed	All females	✓			
T2 - 40 mg/kg feed	All females	✓			
T3 - 60 mg/kg feed	All females	✓			
T4 - 80 mg/kg feed	All females	✓			
T5 - 100 mg/kg feed	All females	✓			

3.4.2. Experiment 1: Effects of DES on survival rates of *G. holbrooki* exposed during the embryonic developmental stage

The mean survival rate (MSR) of both C1 (74.76±15.37%) and C2 (71.26±18.5%) control groups were significantly higher than the treatments groups (Fig. 3.4). Within the treatment groups, the highest MSR was observed in T2 (30.15±6.05%) followed by T1 (26.55±5.58%) and T3 (21.3±5.14%) though not statistically significant. The survival rate for the only clutch of T4 was 20%. Juveniles exposed to the highest DES concentration during the embryonic stage (T5, 100 mg/kg), displayed the lowest (18.26±5.55%) MSR. One-way ANOVA analysis revealed that there was a significant difference in the MSR between the control and treatment groups ($F=16.79$; df: 6, 15; $P<0.05$). In contrast, there was no significant differences between the hormone treatment groups ($P>0.05$).

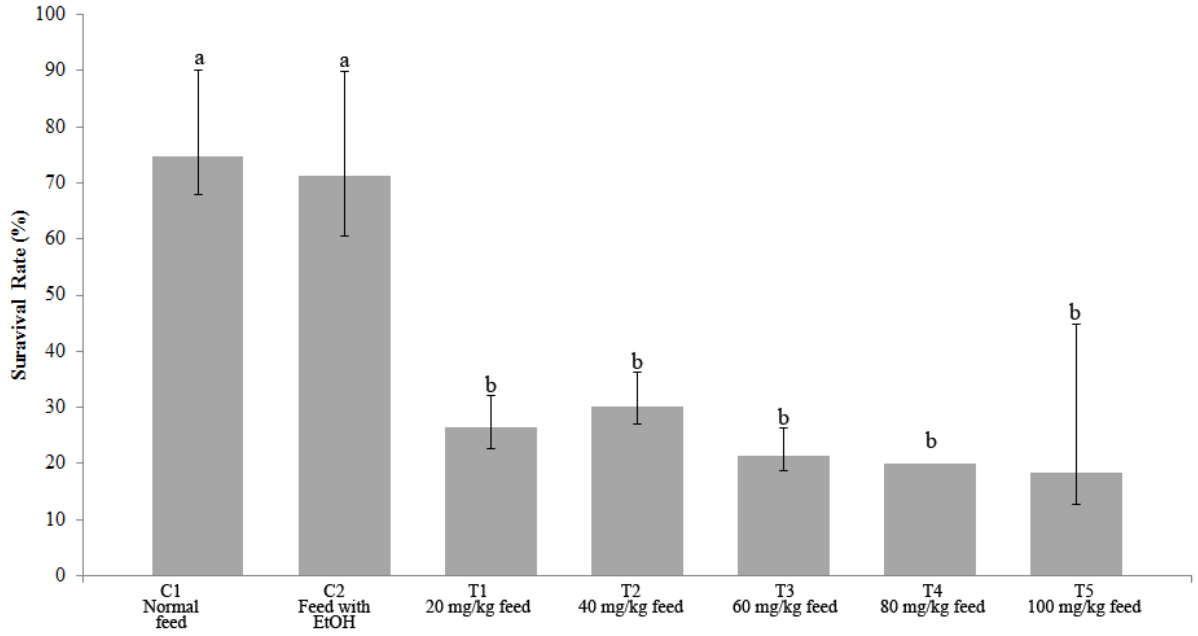


Fig. 3.4. Mean survival rate (mean ± SD) at 30 DAP of juveniles treated with DES during embryonic life stages, compared with controls. Groups with the same superscript were not significantly different from each other ($P>0.05$).

3.4.3. Experiment 2: Effects of DES on survival rates of *G. holbrooki* exposed during the juvenile stage

As in Experiment 1, the MSR of both control groups were higher than the treatments groups where controls were 78.42 ±22.0% and 73.8 ±20.22% for C1 and C2 respectively (Fig. 3.5). Among all the treatment groups in experiment 2, the highest MSR (though not significantly different) was observed in the lowest DES treatment (T1, 20 mg/kg) group (45.6 ±17.93 %) followed by T2 at 40.94±30.0%. The MSR of juveniles in T4 and T5 groups were noticeably lower at 27.94±41.33% and 26.86 ±41.82% respectively. The lowest survival rate was observed in T3 juveniles (18.74 ± 19.0%). The MSR of juveniles for all treated groups and controls was significantly different ($F=3.216$; df: 6, 28; $P<0.05$). However, there were no significant difference between the treatment groups ($P>0.05$).

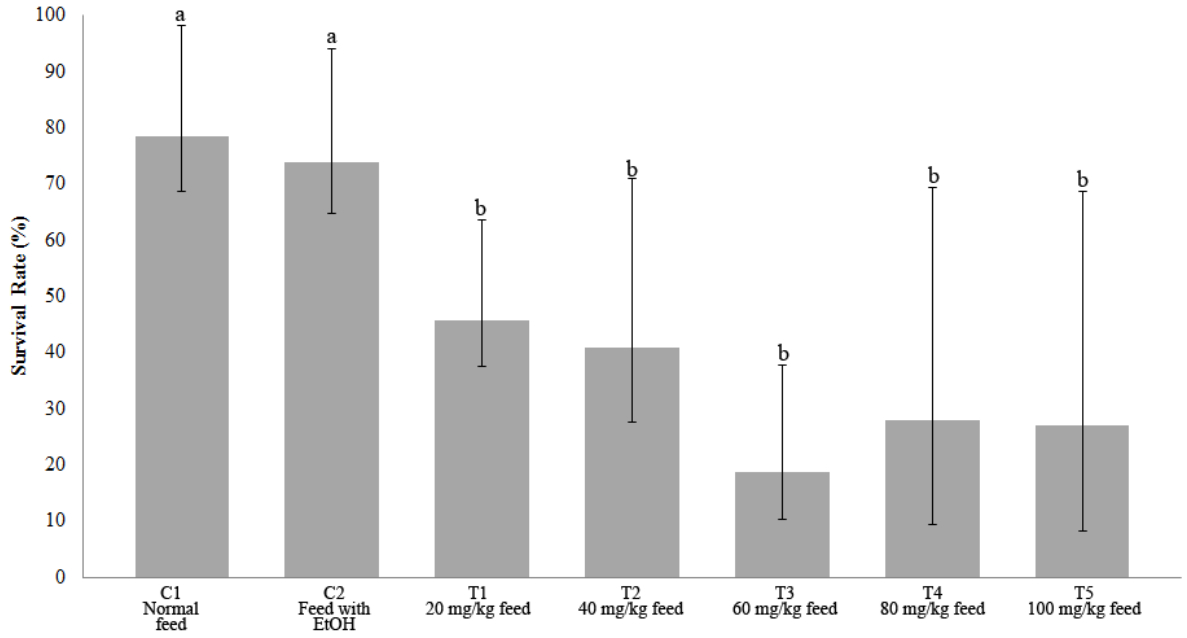


Fig. 3.5. Survival rates (mean ± SD) at 30 DAP of juveniles exposed to a range of DES concentrations compared to controls. Groups with the same superscript were not significantly different from each other ($P>0.05$). High standard deviation is due to the variation in the rearing densities.

3.4.4. Effects of DES on sex ratio of *G. holbrooki*

The sex ratios of juveniles examined at 50 DAP for controls and treatment groups of both experiments are shown in Table 3.2. All the treatment groups produced 100% male fish based on the elongation of the third and fourth ray of the anal fin. The Chi-square analysis conducted on control groups of both experiments showed that they were not significantly different from the expected 1:1 (male:female) sex ratio (Table 3.3). Further observations at 365 DAP on the fish from the treatment groups showed that they had a shorter under-developed gonopodium (Fig. 3.6A) compared to control and normal males (Fig. 3.6B). The tip of the gonopodium in the treatment fish from both experiments lacked the serrated hook structure (Fig. 3.6C) compared to normal and control males (Fig. 3.6D).

Table 3.3. Percentage of sex ratio in control and treatment groups of both experiments at 50 DAP

Experiment	Treatments	Sex Ratio (%)		χ^2	df	P
		Male	Female			
Experiment 1	C1	56.5	43.5	1.673	1	>0.05
	C2	56.3	43.7	1.440	1	>0.05
	T1	100	-			
	T2	100	-			
	T3	100	-			
	T4	100	-			
	T5	100	-			
Experiment 2	C1	44	56	1.440	1	>0.05
	C2	55.5	44.5	1.198	1	>0.05
	T1	100	-			
	T2	100	-			
	T3	100	-			
	T4	100	-			
	T5	100	-			

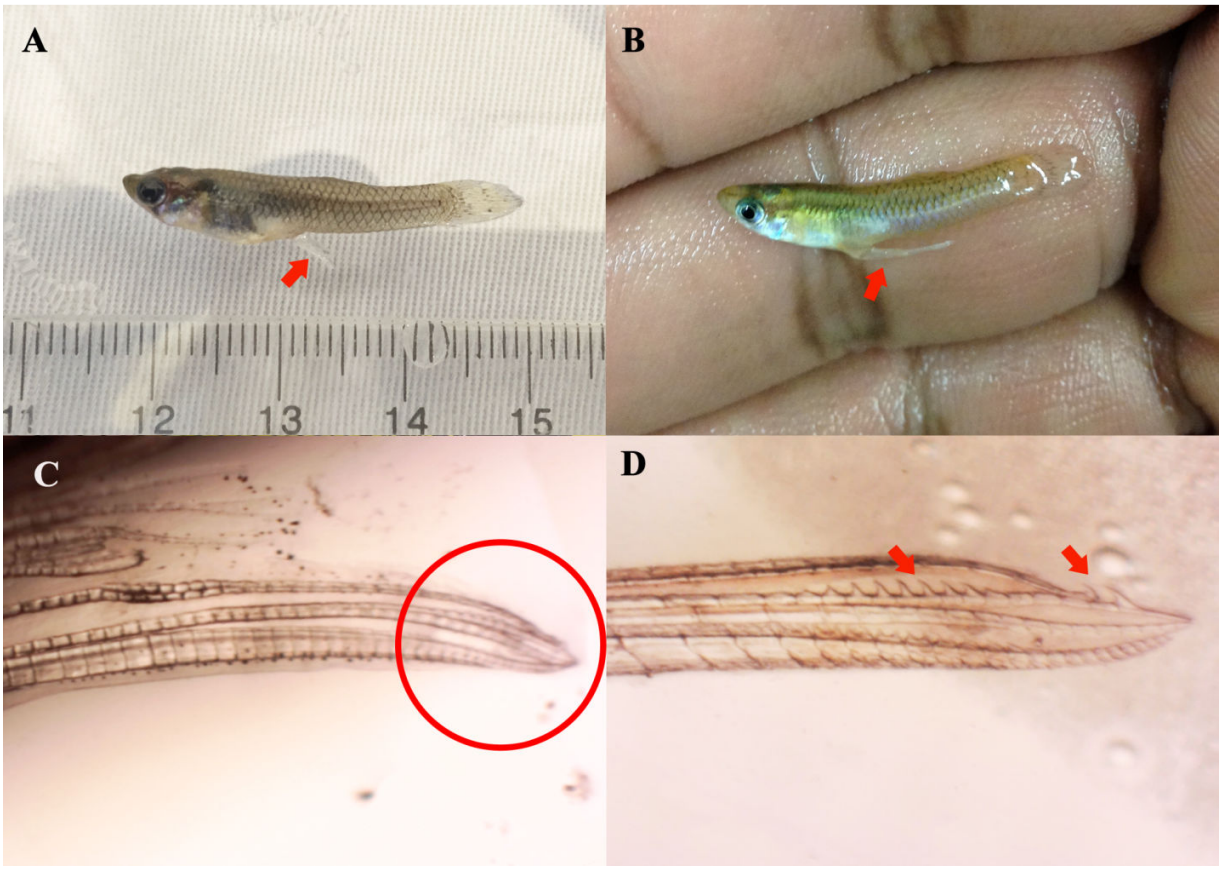


Fig. 3.6. Representative photograph of *G. holbrooki* gonopodium at 356 DAP (A) Significantly shorter gonopodium observed in all DES treated fish in both experiments (red arrow); (B) Gonopodium of normal and control group males (red arrow); (C) Absence of serrated hooks (circled red) at the tip of shortened gonopodium in DES treated individuals; and (D) Hooks at the tip of a mature control male gonopodium.

3.5. Discussion

Diethylstilbestrol (DES) is known as one of the most potent non-steroidal estrogens (Piferrer, 2001) that has been commonly used in the aquaculture industry to induce fish sex reversal through the feminization of fish (Pandian and Sheela, 1995). It has been used as a hormone of choice to sex reverse multiple fish species including the livebearing guppy (*Poecilia reticulata*) and black molly (*P. sphenops*) (Kavumpurath and Pandian, 1992, George and Pandian, 1995), both species closely related to *G. holbrooki*. Thus, DES was chosen as a feminising hormone to determine optimum dosage, duration of treatment and the appropriate life stage for sex reversal in *G holbrooki*. In contrast to earlier reports on other livebearers, the current study showed that DES treatment of *G. holbrooki* caused adverse effects on the reproduction (gonadal atrophy), and survival rates of juveniles, and instead of feminising the fish, a paradoxical masculinization effect was observed in treated fish. Here the findings of this study are discussed.

3.5.1. Suitability of DES dose

Compared to other reports, the nominal dosage of DES used in this study (20–100 mg/kg diet) was considerably lower. For example, the dosage of hormone used in the sex reversal of livebearing poecilids is usually between 300-500 mg/kg of feed in guppy and black molly (Kavumpurath and Pandian, 1993b, George and Pandian, 1995, Pandian and Sheela, 1995). However, a preliminary scoping study conducted by the author using DES at 100-400 mg/kg of feed on gravid *G. holbrooki* females showed adverse effects where parturition did not occur and skin haemorrhage were observed in the treated females at 200-400 mg/kg feed (data not shown). Therefore for fish welfare reasons 100 mg/kg was chosen as the maximum dosage for this study.

3.5.2. DES induced atrophy in the ovary of treated females

Of the 35 gravid females used in this study, more than half (n=22) parturated while few (n=7) did not parturate and six did not survive. Examination on the gonads of the two control (C2) females that did not parturate at the end of the 30 day treatment period showed that both females possessed unfertilized mature eggs thus indicating that fertilization did not occur following the first parturition in these individuals. It is known that this livebearing species has the ability to store sperm and use it to fertilise eggs following the birth of the previous clutch (Jobling, 1995, Koya et al., 2003, Bone and Moore, 2007). Noting that the treated and control females were not mated after first parturition, it is possible that the sperm stored in the females (from previous copulation) was completely used in previous fertilization events and therefore resulted in failure of second parturition in these individuals. This might also be the case for those DES treated individuals that did not parturate after the treatment started. It is unlikely that DES itself caused failure of fertilization in treated individuals as the treatment was started 14 days after the first parturition event. Furthermore, the remainder of the females in the treatment groups (except the ones that died) successfully parturated.

Routine observations in our laboratory showed that *G. holbrooki* parturate between one to four times after a single copulation. It is quite difficult to know how many times a female can parturate after copulation occurred since it varies between females (data not shown). The mechanism that allows sperm storage, volume of storage and how the females ration the sperm reserve for subsequent fertilization in this species and other livebearing fish in general is yet to be fully elucidated. A combination of mating, breeding, histological and physiological studies are likely to shed more light in this regard.

The mean gestation period observed (28.9 ±2.97 days) in this experiment is similar to the one reported for control fish in chapter 2 (Norazmi-Lokman et al. (2016). These results suggest that DES treatments on gravid females have not significantly affected the gestation period of *G. holbrooki*. Similar findings were also reported in guppies treated with DES where the gestation period observed was between 23-30 days and comparable to controls (Kavumpurath and Pandian, 1993b).

The observation that the gonad of the treated females following second parturition shrunk suggests that the DES perturbed the re-maturation cycle in the species. It is interesting to note that the females despite the regressed gonad externally exhibited the features of a gravid female —displayed distinct gravid spot intensity despite the small size. This is due to the retention of dark pigmentation on the ovarian sac which has been known to impart the characteristic features to the gravid spot (Norazmi-Lokman et al., 2016), with reduced spot size attributed to ovarian atrophy in the treated individuals.

There are several causes for gonadal atrophy in fish which includes parasite infection (Parsa Khanghah et al., 2011) and exposure to heavy metals (Friedmann et al., 1996), other toxic contaminants (Lehmann et al., 2007) and endocrine disruption compounds (EDC). Specifically male carp exposed to polluting EDCs are known to undergo testicular atrophy (Solé et al., 2003) while exposure to estrogenic EDC in a polluted lake caused alteration in the oogenesis of female fathead minnow (*Pimephales promelas*) (Kidd et al., 2007).

Specifically, DES was found to cause ovarian atrophy and malformation in catfish (*Clarias gariepinus*) immersed in a range of dosages (1µg/L -10ng/L) from hatch to 50 DAH (Sridevi et al., 2015). The report indicates that DES altered the transcript level of steroidogenic enzymes which disturbed the estrogen production and its level in catfish where this can affect the downstream estrogen-dependent processes (Cheshenko et al., 2008). However, since DES only alters the expression of *cyp19a1b* (brain aromatase); the report (Sridevi et al., 2015) infers that it is also possible that the adverse effect on the gonad is caused by DES without altering the expression level of c*yp19a1a* and estradiol (E2) levels in the ovary. Meanwhile in medaka (*Oryzias latipes*), prolonged treatment (18-28 days post fertilization) of DES (0.01-100 ng/ml) during the embryonic stages affected germ cell mitosis which reduced the number of germ cells to more than half leading to reduced gonad weight in adulthood (Paul-Prasanth et al., 2011). The mechanism underlying the negative response of mitotic activity of germ cells in medaka to DES is still unknown. Interestingly, the study also reports that long DES exposure suppress the production of c*yp19a1* (gonad aromatase) in medaka which indicates that endogenous E2 was not produced, in the gonad. Beside fish, adverse effects of DES have also been reported in mice where testicular atrophy and a reduced number of mature oocytes along with altered expression level of various genes expressed in the liver, kidney, ovary and testis was discovered after exposure to DES via injection (5µg/body weight) (Hong et al., 2010). On the contrary, it was reported that *in vitro* DES exposure triggers oocyte maturation in zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*) where its mode of action on the oocyte is similar to those of the natural maturation-inducing hormone; 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) (Tokumoto et al., 2004). Therefore the ex-vivo administration of DES affects and disrupts endocrine and physiological activities at both molecular and cellular levels both activating and

repressing steroid biosynthesis, that are not in tune with endogenous feedback regulatory mechanism—manifesting in the observed gonad atrophy.

In fish, E2 is responsible in stimulating the liver to produce the yolk protein vitellogenin which plays a major role in oocyte development hence directly affecting gonadal weight during recrudescence (Kime, 1998). Conceivably, DES inhibited the production of E2 by repressing the expression of aromatase gene in gravid *G. holbrooki* as observed in medaka (Paul-Prasanth et al., 2011) thus affecting endogenous E2 production. Diethylstilbestrol than binds with estrogen receptors at high affinity (Blair et al., 2000) in the liver but its minimal quantity (since low DES concentration was used in this study) might have affected the low production of vitellogenin by the liver thus causing the gonadal atrophy (Fig. 3.7). Another possible endocrine pathway that might be affected by DES thus causing gonadal atrophy is the hypothalamic-pituitary-gonadal (HPG) axes (Wishart et al., 2006). By binding with estrogen receptors, DES enters the hypothalamus and pituitary consequently suppressing the secretion of gonadotrophin releasing hormone (GnRh) and gonadotrophin hormones (GtH) particularly the follicle stimulating hormone (FSH) (Wishart et al., 2006), which will inhibit the development and maturation of oocytes and their secretion of steroids (Kime, 1998). The suppression of GtH will also adversely affect the uptake of vitellogenin by oocytes in the vitellogenesis pathway thus leading to gonadal atrophy (Kime, 1998). Nonetheless, molecular experimentation needs to be conducted in *G. holbrooki* to gain a better understanding of the mechanics behind the ovarian atrophy that was caused by DES.

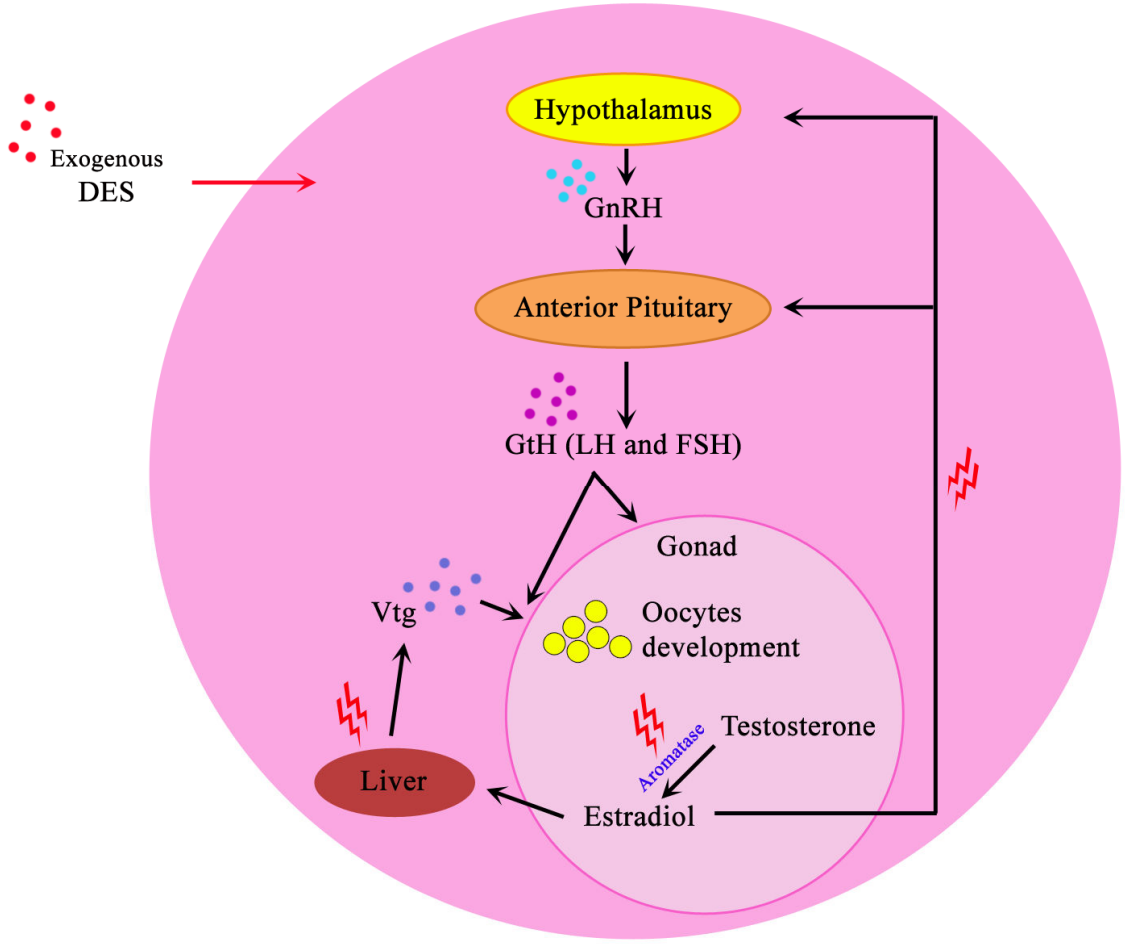


Fig. 3.7. Hypothalamic-pituitary-gonadal (HPG) axes and vitellogenesis pathway in fish adapted from Kime (1998) showing potential sites of DES disruption/interaction. Red arrow indicates ex-vivo administration of DES and its target pathways and organs are marked with double lightning rod. (DES: Diethylstilbestrol; GnRH: Gonadotrophin releasing hormone; GtH: Gonadotrophin hormones; LH: Luteinizing hormone; FSH: Follicle stimulating hormone; Vtg: Vitellogenin)

3.5.3. Adverse effect of DES on survival rates

Survival rate of the treated individuals in conjunction with efficacy of sex reversal serve as indicators to assess optimum treatment dose. As observed in the current study both embryo (via mothers) and newborn exposures yielded very low survival rates. Therefore, at the administered doses, the DES appears to be an unsuitable feminising agent in this pest fish. The possibility of DES might be selectively lethal to females was unlikely due to the overall low survival rate in exposed fish. If this was the case, the expected survival rates should be about 50% of controls, (assuming 50:50 male:female sex ratio). However, the highest survival rate was only 40% suggesting that DES killed both male and female fish. The sex specific mortality/toxicity or the diametrically opposed action of the DES in the two sexes cannot be completely confirmed at this stage. Further studies exploring the effects of lower doses of DES are necessary before it can be completely discounted.

In contrast, the survival rate of black molly offspring treated at comparable doses of DES (between 25-300 mg/kg food) was much higher (highest survival rate was 88% for the lowest dose and 50% for the highest dose) (George and Pandian, 1995) compared to this study (i.e. in E2: highest MSR was 45.6 ±17.93 % at lowest dose and lowest MSR was 26.86 ±41.82 % at the highest dose). Apart from these studies, there are no additional reports on the survival rates of livebearing fish that could be directly compared with juveniles in Experiment 1 of this study. Similarly, immersion treatment in catfish at higher doses (1µg/L -100ng/L), resulted in low survival rates (between 0-10%) whilst the lowest dosage (10ng/L) yielded higher survival rate of 90% and also resulted in sex reversal (Sridevi et al., 2015). On the contrary, the survival rate of

juvenile European catfish (*Silurus glanis*) was high (>85%) for all treatments (15 – 60 mg/kg feed) although it was lower compared to controls (97.5 %) (Król et al., 2014).

The MSR of the treatment groups in Experiment 1 was lower than Experiment 2. It therefore appears that the embryonic stages are more susceptible to the adverse effects of DES than the juveniles. Given the longer exposure period and relatively unregulated level of DES ingestion by the juveniles (ingestion level might be different among individuals); one would expect the MSR of the treatment groups in Experiment 2 to be lower. There might be other aspect or mechanism during embryonic developmental progress that was negatively affected by the DES treatment that is yet to be known.

3.5.4. DES causes paradoxical masculinization

Contrary to expectations of feminisation, all treatments resulted in production of 100 % males (masculinisation) implying a paradoxical effect of the hormone at all the doses administered. Paradoxical effect of hormonal sex reversal is not uncommon and has been reported not only in fish but also in amphibians and reptiles (Piferrer, 2001). In teleosts, it has been reported to occur in coho salmon (*Oncorhynchus kisutch*) (Piferrer and Donaldson, 1991), channel catfish (*Ictalurus punctatus*) (Davis et al., 1990), bluegill (*Lepomis macrochirus*) (Al-Ablani and Phelps, 2002), medaka (Iwamatsu et al., 2006) and false clownfish (*Amphiprion ocellaris*) (Abduh, 2010). It is also possible that the rearing conditions, particularly temperature may have contributed to the paradoxical effect as it (temperature) is known to influence sex ratios in fish (Devlin and Nagahama, 2002). However this is unlikely, as the control groups displayed the expected sex ratios. It has also been reported that ethanol causes masculinization in genetically

female *G. affinis* (Senior, 2013). Nevertheless, in the current study, this possibility can also be ruled out as the sex ratio of carrier (ethanol) controls (C2 group) in both experiments was not altered suggesting that DES at the treated doses was the primary cause of the paradoxical masculinisation observed.

Paradoxical effect is known to occur as a result of exposure to an aromatizable androgen at high concentration or over a prolonged treatment period and has been termed as paradoxical feminization (Piferrer, 2001). However, there have been no reports of paradoxical masculinization caused by estrogen exposure in fish and it was believed that there is no apparent mechanism for such an effect (Piferrer, 2001). Interestingly, paradoxical masculinization has been reported in *G. affinis* treated with spironolactone, a drug that is known to have anti-androgenic properties but the mechanistic action that caused such an effect by the drug is yet to be known (Howell et al., 1994, Raut et al., 2011). This study is the first to report the paradoxical masculinization caused by synthetic non-steroid estrogen in fish. Similar estrogen-induced paradoxical masculinisation effect has also been reported recently in two turtle species; the painted turtle (*Chelydra picta*) and the common snapping turtle (*C. serpentina*) that were exposed to E2 via exogenous application to the eggs (Warner et al., 2014). The report suggests that the abnormally high E2 concentration above physiological levels might have interacted with androgen receptors or inhibited aromatase expression thus resulting in high testosterone concentrations in the system leading to masculinization. This could be a possible reason for the paradoxical masculinization observed in this study.

Biosynthesis of natural estrogen (Fig. 3.8) from androgen in the body is regulated by the aromatase gene (Piferrer and Blazquez, 2005). Endocrine disrupting compounds are known to alter aromatase gene expression level disrupting the mechanism of sexual differentiation and reproduction in fish (Cheshenko et al., 2008). For example, a report in medaka has shown that the exposure to exogenous DES inhibits the production of endogenous E2 by repression of *cyp19a1* gene which encodes aromatase (Paul-Prasanth et al., 2011). This might result in the accumulation of testosterone leading to the masculinization effect observed in both experiments in this study (Fig 3.8). In addition, there are also possibilities that DES disrupts the synthesis of E2 in the gravid female's gonad in Experiment 1 which increased the gonad testosterone concentration thus masculinizing the embryos (progenies) in the ovarian sac.

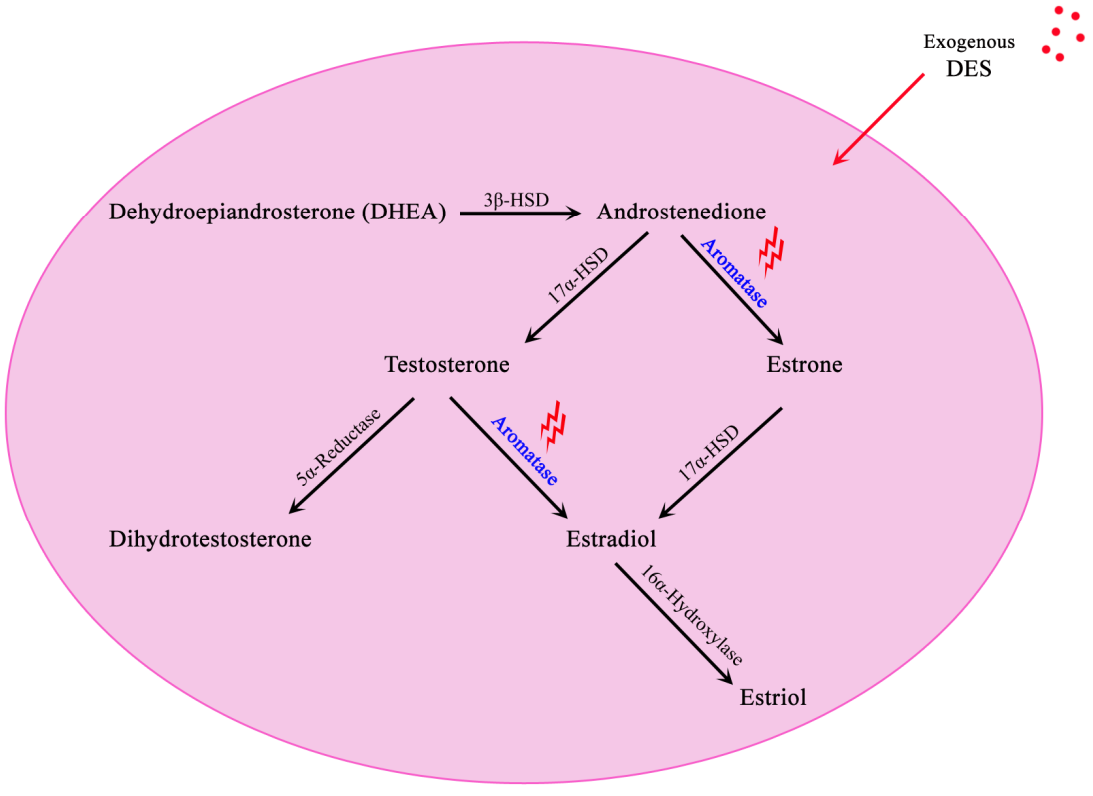


Fig. 3.8. Gonadal biosynthesis of androgen and estrogen adapted from Kime (1998) and Palmer et al. (2012). Red arrow indicates ex-vivo administration of DES while the pathways of E2 synthesis it disrupts are indicated by the red lightning rod.

Although, the concentrations of DES used in this study are low and the exposure period is quite short, there is likelihood that DES can regulate the aromatase activity via feedback loop. For example in guppy, a short period of exposure (12-14) days to 17α-ethynylestradiol (an estrogen that has almost the same potency as DES) at environmentally relevant concentrations altered the brain aromatase activity where the activity levels were higher in exposed male fish compared to controls (Hallgren and Olsén, 2010). It is also therefore, possible that DES could repress aromatase activity via feedback causing the paradoxical masculinization as was observed in this study. However, further studies are warranted to demonstrate the repression of aromatase activity following DES treatment in *G. holbrooki*.

Long exposure of medaka embryos to DES inhibited germ cell mitotic activity where it reduced (>50%) the number of germ cells in XX embryos (Paul-Prasanth et al., 2011). If this is also the case in *G. holbrooki*, it might also change the course of sex differentiation from female to male in its undifferentiated gonad due to low germ cell proliferation (Martínez et al., 2014) (Fig. 3.9). Furthermore, if E2 were suppressed by DES during *G. holbrooki* gonad differentiation (Fig. 3.8), this will then conceivably further induce testis differentiation. Future molecular and cellular analysis in treated fish will be valuable to provide insights and explain this paradoxical effect in *G. holbrooki*.

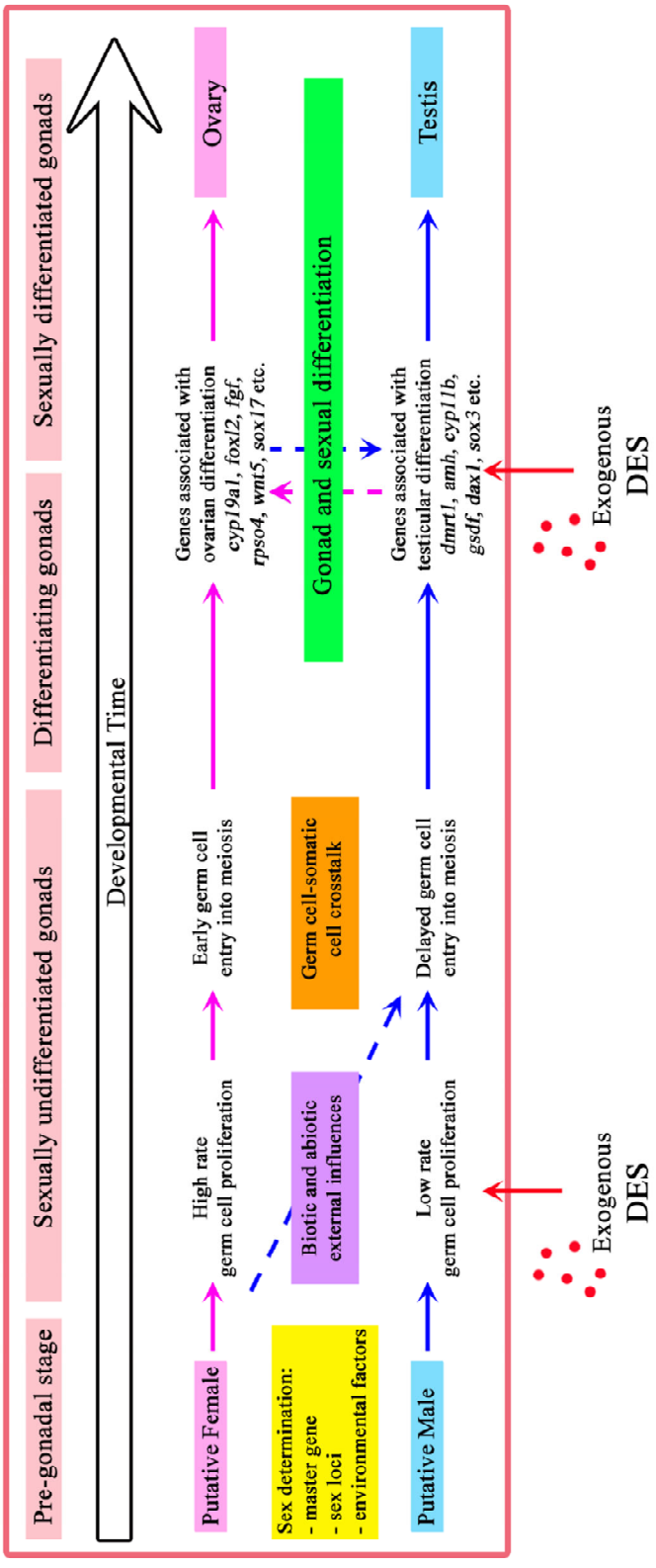


Fig. 3.9. Major events leading up to gonad differentiation (adapted from Martínez et al. (2014)). Exogenous DES might affect mitotic activity in genetic female of *G. holbrooki* thus changing the direction of gonad differentiation from female to male (left red arrow) and also affect the synthesis of E2 during gonad differentiation (as in Fig. 3.8) causing testosterone accumulation in gonad (right red arrow). These entire events conceivably lead to the paradoxical masculinization effect observed in this study.

3.5.5. Could leaching and accumulation of DES in rearing water cause overexposure resulting in the paradoxical effect?

Each treated gravid females in Experiment 1 consumed an average of 300 mg of hormone enriched feed while the newborn juveniles consumed an average of 12 mg feed throughout the 30 days treatment period. This means that the actual quantity of DES delivered to the treated fish during the 30 days treatment period are different (lower) from the nominal DES dose (see Appendix 3). Despite the low quantity of DES delivered to the treated fish in this study, there is a likelihood that a considerable amount of DES leached from the food and faeces which accumulated in the rearing water, inadvertently overdosing the fish through skin immersion. During treatments of gravid females in Experiment 1, some of the feed sank straight to the bottom of the tank outside the breeding trap thus were left uneaten. The amount of leftover feed and faeces was not recorded and syphoned out between the two days water change interval to avoid stress to the females that might occur if they were frequently moved out of the breeding trap. In Experiment 2, although feeding was stopped when the juveniles feeding response ceased, it was difficult to determine how much food was left in the rearing tank due to the small size of the spirulina powder. Since the rearing water was only changed every two days, this might have caused overexposure. However, the standard water quality parameters remained within acceptable limits.

A report on tilapia reared in an unfiltered system has shown that even after the fish had consumed all the methyltestosterone (MT) enriched feed, the level of MT remained high in the rearing water for 30 minutes and was still detectable after 15 hours (Sánchez, 2001). If overexposure due to leaching occurred, the excessive level of DES might affect the endocrine

activities in treated fish as discussed in the previous section. Since the quantity of delivered DES/hormone can be estimated by calculating the dosage and amount of hormone enriched food consumed by the fish, it is recommended that future fish sex reversal experiment that involves oral hormone administration includes hormone analysis of the rearing water in their study so that the total hormone exposure the fish had can be ascertained.

3.5.6. Short gonopodium in DES treated *G. holbrooki*

Typically, both sexes of Gambusia are born with an undifferentiated anal fin where genetically male and female fish possess the same anal fin structure prior to puberty. The anal fin later develops and elongates during maturation in male Gambusia (Turner, 1941) since the formation of the anal fin is androgen dependent (Ogino et al., 2004). Exposure to estrogenic EDCs has been shown to supress the development of the gonopodium in genetic male juveniles (Doyle and Lim, 2002, Doyle and Lim, 2005, Angus et al., 2005, Rautenberg et al., 2015) while stimulating its development in females (Howell et al., 1980, Angus et al., 2001).

Assuming a natural genetic sex ratio of 1:1 (male:female), the observed shortening of the gonopodium of all treated progeny (at 365 DAP) suggests a diametrically opposed action of the hormone on genetic male and female fish in the groups i.e. the DES supressing and promoting the development of gonopodium in genetic males and females respectively. The gonopodium was only checked at 365 DAP after experiments related to E2 treatments (chapter four and five) were done since the successful feminization of *G. holbrooki* with estradiol prompted us to re-examine the condition of DES treated fish.

3.6 Conclusion

Based on the observation of paradoxical effects, DES at the administered doses appears less suitable for feminisation in *G. holbrooki*. Not only does it not produce sex reversed females, DES also exhibits adverse effects particularly on survival and reproduction (gonadal atrophy and under-developed gonopodium) in the species. A more detailed experiment, using lower dosages of DES, could yield the desired feminising effects. Further studies at molecular and cellular level are also warranted to provide mechanistic understanding of the ovarian atrophy and the rare paradoxical masculinisation effect observed in this study. Nevertheless, the findings of this study will be beneficial for future eco-toxicological studies related to EDC pollution using *G. holbrooki* as a bio-indicator.

CHAPTER 4

Efficacy of Estradiol on Sex Reversal in the Eastern Mosquitofish (*Gambusia holbrooki*)

4.1. Abstract

As part of the Trojan Sex Chromosome (TSC) strategy to eradicate the introduced pest fish *Gambusia holbrooki*, this study was carried out to investigate the efficacy of Estradiol (E2), a commonly used estrogen in the aquaculture industry, to produce a feminized *G. holbrooki* population. The estrogen was administered orally to two groups of *G. holbrooki* at different life stages: (Experiment 1) embryonic development stage through gravid females and (Experiment 2) newborn juveniles. The dosage of E2 ranged between 50-400 mg/kg of feed. Two control groups were set for each experiment: (C1) normal feed (no chemical exposure) and (C2) feed mixed with 70% ethanol (vehicle control). In Experiment 1, a 100% female population was achieved at the three E2 concentrations of 200, 300 and 400 mg/kg. Among the three concentrations, the best survival rate was displayed in the 200 mg/kg concentration with juvenile mean survival rate (MSR) of 59.33±12.54%. One-way ANOVA analysis found a significant difference ($F=4.38$; df: 6, 27; $P<0.05$) between the juveniles MSR of all the treatment and control groups (C1: 79.96 ± 20.33%; C2: 77.09 ± 10.32%). In Experiment 2, 100% feminization of fish was observed at all E2 concentrations except at 400 mg/kg where no treated individuals survived. Statistical analysis shows that there was also a significant difference ($F=7.27$; df: 5, 24; $P<0.05$) between the MSR of all E2 treated juveniles and controls (C1: 71.73 ± 22.86%; C2: 70.02 ± 18.26%) with those treated at E2 concentration of 50 mg/kg showed the best MSR (66.38±12.34%) among the treated fish. The study provides a framework for reliable sex reversal (feminisation) in this fish paving the way for developing TSC strategy to control and eradicate this noxious pest.

Keywords: Gambusia, Invasive fish, Livebearer, Endocrine Disrupting Compound, Feminization.

4.2. Introduction

Hormone sex reversal is a technique that has been widely used to manipulate sex ratios in fish. With the successful synthesis of estradiol (E2) in the late 1930's (Piferrer, 2001), research on this technique gained worldwide scientific interest. Due to the diverse and labile nature of the sex differentiation process (Devlin and Nagahama, 2002), together with their economic and social importance, fish have been the main species where the hormone sex reversal technique has been studied, developed and applied as evidenced by the abundance of related literature. Records show that there are more than 200 reports that have been published on hormone-induced sex reversal in fish (Senior and Nakagawa, 2011).

In the aquaculture industry, sex reversal is mainly used in the production of a monosex population. In some fish species, the sex that displays certain favourable traits is much more desirable than the other. For example, female cyprinids and salmonids show better growth traits compared to males (Bartley and Subasinghe, 2005) while in most ornamental fish species, male fish display more attractive colour and patterns (Piferrer and Lim, 1997). Besides that, monosex fish population also facilitates in stock management programs, for example, when there is a need to control or inhibit breeding. In the case of the hermaphrodite false clownfish (*Amphiprion ocellaris*), hormone sex reversal was applied to captive-produced juveniles (fish with immature ovotestis) to induce a sex change for broodstock development and management thus increasing the production in captive conditions (Abduh, 2010, Thuong, 2010). Hormone sex reversal is also used in fisheries management for the purpose of producing sterile fish (Donaldson and Hunter, 1982) useful in protecting wild populations and aquatic environment biodiversity in the event that captive-reared fish escape to the wild. Recently, hormone sex reversal has also been

proposed for managing aquatic invasive alien species (IAS) via the Trojan Sex Chromosome (TSC) Strategy (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007).

The TSC strategy involves the release of individuals carrying the sex chromosome of the opposite phenotype—produced via hormone sex reversal—into the wild to cause population extinction (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007, Stelkens and Wedekind, 2010). This strategy has been identified as a potential alternative approach to eradicate the eastern mosquitofish, *Gambusia holbrooki* (Patil, 2012), an introduced livebearer that has been declared a noxious pest species in Australia (NSW National Parks and Wildlife Service, 2003). Although hormone sex reversal has been successfully achieved in other livebearing species such as guppies (*Poecilia reticulata*), black mollies (*P. sphenops*) and the western mosquitofish (*G. affinis*), (Kavumpurath and Pandian, 1993b, George and Pandian, 1995, Senior, 2013), no reports can be found on *G. holbrooki*. In order to apply the TSC strategy in *G. holbrooki*, a protocol to feminize this species needs to be developed. Focus needs to be directed on *G. holbrooki* feminization since this species possesses the XX-XY sex determination (Angus, 1989a, Angus, 1989b, Horth, 2006, Horth et al., 2013) system thus production of YY females as Trojan chromosome carriers is necessary (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007, Senior et al., 2013). The experiments in the previous chapter (chapter three) demonstrated that DES caused paradoxical masculinisation instead of feminisation in *G. holbrooki*; hence E2 was tested in this chapter.

Estradiol (17β-estradiol; oestradiol; E2) is the most widely used natural estrogen in fish feminization. Compared to the other two natural estrogens; estrone (E1) and estriol (E3), E2 is

the most effective hormone in fish feminization (Piferrer, 2001). Although synthetic estrogens such as 17α-ethynylestradiol (EE2) and diethylstilbestrol (DES) are more potent than E2, the use of these synthetic hormones is controlled under specific regulations in certain countries such as the European Union (Piferrer, 2001) thus making E2 preferable among scientists and aquaculturists. Estradiol has been used in the sex reversal of more than 40 fish species including swordtails (*Xiphophorus helleri*) (Lim et al., 1992), chinook salmon (*Oncorhynchus tshawytscha*) (Piferrer and Donaldson, 1992), guppy (Kavumpurath and Pandian, 1993b), black molly (George and Pandian, 1995), Atlantic halibut (*Hippoglossus hippoglossus*) (Hendry et al., 2003), western mosquitofish (Senior, 2013) and brook trout (*Salvelinus fontinalis*) (Schill et al., 2016) via oral, immersion and injection methods (Piferrer, 2001).

In hormone-induced sex reversal of fish, information on hormone suitability, treatment dosage, route of administration and the appropriate life stage for treatment are crucial to ensure efficient and feasible hormone administration. Towards the development of a sex reversal protocol for *G. holbrooki* as part of the TSC approach, this study investigated:

1. The effects of E2 on the reproductive traits/outputs of the treated gravid females;
2. The effects of E2 on the survival of treated embryos (via ingestion by gravid females) and newborn juveniles (direct feeding) and;
3. The efficacy of E2 oral administration in feminizing *G. holbrooki*.

4.3. Materials and Methods

This study was carried out based on the reproductive biology and behaviour information obtained from Norazmi-Lokman et al. (2016) (chapter two) and closely follows the experimental design used in chapter three when testing the efficacy of DES (chapter three). Similar to DES, the E2 was administered to *Gambusia holbrooki* at two different life stage groups: (i) embryonic stage through gravid females (Experiment 1) and (ii) newborn juvenile stage (Experiment 2). Both experiments were run concurrently.

4.3.1. Source of Specimens

The stock of *G. holbrooki* in this study was collected during summer in the first week of January 2015. They were captured using dip nets at the Tamar Island Wetland Reserve (TIWR), Launceston, Tasmania and were transported to the Institute for Marine and Antarctic Studies (IMAS) Aquaculture Centre, University of Tasmania, Newnham, Tasmania. Fish were acclimatized in a recirculating aquaculture system and fed twice daily to satiation with commercial fish pellets (TetraMin® tropical granules, Germany). After a week, 35 gravid *G. holbrooki* females (size range: 31.0-46.0 mm TL) were chosen from the stock tank. All gravid females carried embryos at late developmental stages and displayed a gravid spot intensity value between 28-38 (Norazmi-Lokman et al., 2016). Static tanks (2.5L) fitted with a breeding trap were used to house each female separately where they were then left to parturate. Juveniles obtained from the first parturition were separated from the females and transferred into a different static rearing tank (1.5l). These newborn juveniles were used for the second experiment while the females were used in the first experiment. A static tank system was used in both

experiments to avoid cross contamination, especially of control tanks by E2. Each static tank was supplied with gentle aeration and water quality was maintained within acceptable limits by water exchanges which were undertaken every two days using aged and aerated tap water. Throughout this study, the rearing temperature was set at ±25°C, salinity at 0ppt and the photoperiod was 16L : 8D (lights on at 06.00h).

4.3.2. Preparation of E2-enriched feed

Commercial fish feed was used as the carrier of E2 (CAS No.: 50-28-2; Novachem, Australia) where it was mixed with Tropical fish granules (TetraMin®, Germany) for treatments on gravid females while powdered spirulina (Bioglan, Australia) was used on the newly born juveniles. The dosage of E2 for both experiments were set at 50, 100, 200, 300 and 400 mg/kg diet (labelled as T1 – T5 respectively) based on studies conducted in guppy *(Poecilia reticulata*) and black molly (*P. sphenops*) (Kavumpurath and Pandian, 1993b, George and Pandian, 1995). Since preliminary studies indicated that DES cause skin lesions at 100 mg/kg (chapter three), a lower dosage of 50 mg/kg of E2 was also included as a safeguard. There were two control groups in this study; Control 1 (C1): normal feed (no chemical exposure) and Control 2 (C2): feed mixed with 70% ethanol (vehicle control). The determined amount of E2 for each treatment was first dissolved in 20ml of 70% ethanol before it was mixed thoroughly with 20 g of each feed item. After being spread into thin layers on a tray, the E2-enriched feed was allowed to dry for 24 hours at room temperature in a fume hood. All the feed items were then kept in sealed containers and stored at ±4°C (Phelps and Popma, 2000, Angus et al., 2005,).

4.3.3. Experimental Design.

The experimental design and overall workflow of both experiments are shown by Fig 4.1. Both experiments were conducted concurrently.

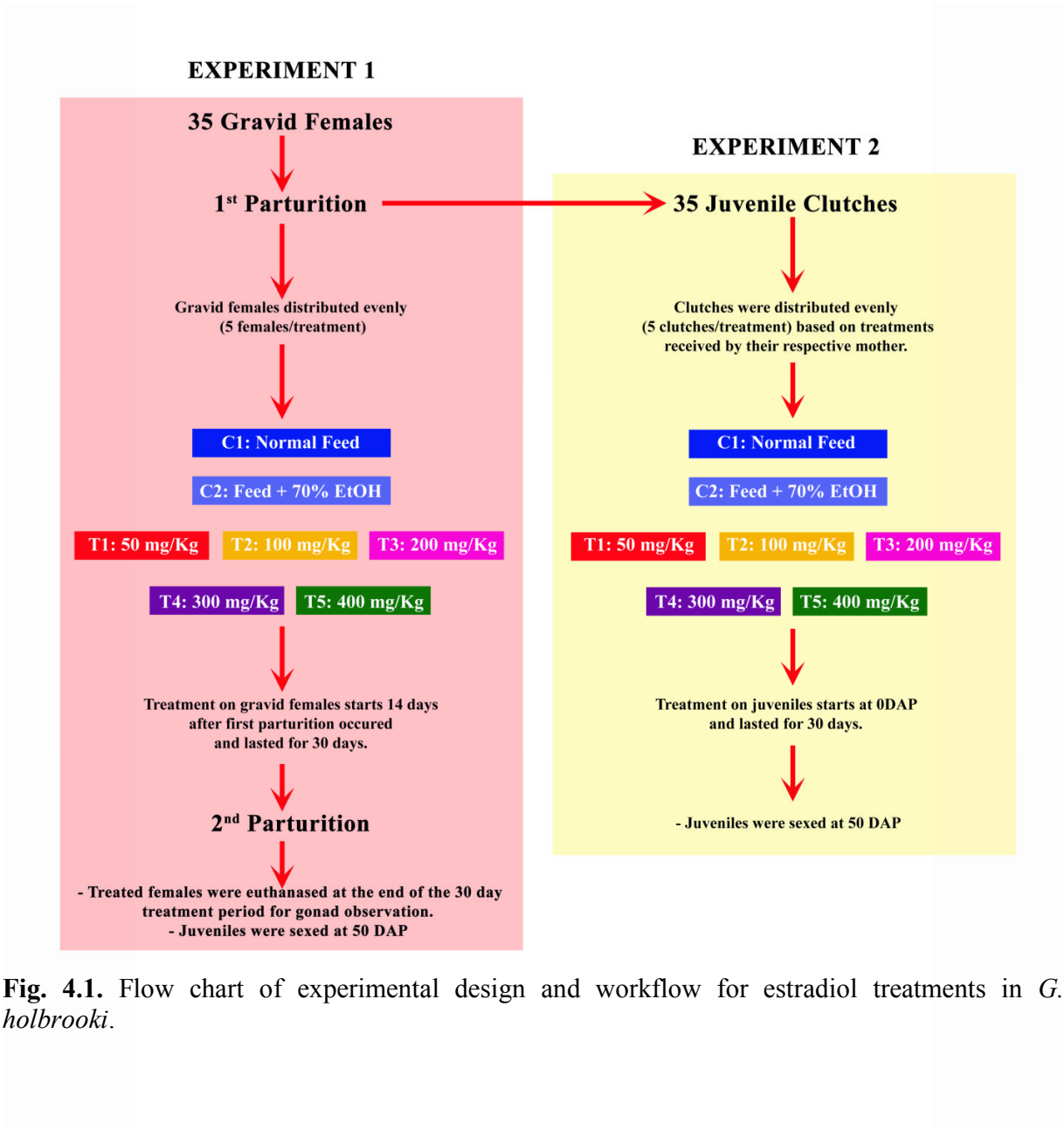


Fig. 4.1. Flow chart of experimental design and workflow for estradiol treatments in *G. holbrooki*.

4.3.3.1. Experiment 1: E2 treatment on gravid *G. holbrooki* females

Five gravid females with gravid spot intensity values between 28-38 (Norazmi-Lokman et al., 2016) were used in each treatment and control groups. They were allowed to parturate and the newborn juveniles were then transferred as a clutch to separate tanks (for Experiment 2). The females were fed with control diets for 14 days after the first parturition to allow them to prepare for the next reproduction cycle (Norazmi-Lokman et al., 2016). After the 14 days, E2-enriched diets were administered twice daily (morning 0900-1000h; evening 1600-1700h) to satiation (≈5% body weight per meal). Duration of the treatments was 30 days. Progeny produced following parturition were transferred and reared in a static tank (1.5L, 0ppt, ±25°C) in a separate group. Commercial powdered spirulina (Bioglan, Australia) was fed to the progeny for 30 days before they were weaned over 10 days onto commercial micropellets (Hikari®, Japan). The survival rates of the juveniles were recorded and fish were sexed at 50 days after parturition (DAP) using secondary sexual characters (anal fin morphology and appearance of the gravid spot) (Angus et al., 2005). At the end of the treatment, all the females were humanely euthanased by overdosing with benzocaine (1:2000) for microscopic gonad morphology observation. Gestation period was measured and embryos were staged as per the method in Norazmi-Lokman et al. (2016).

4.3.3.2. Experiment 2: E2 treatment of *G. holbrooki* newly born juveniles

The clutches of newly born juveniles obtained from the first parturition were used in this experiment. They were administered with E2 enriched spirulina from 0 until 30 DAP based on the dose received by their respective mothers in the first experiment. The E2 enriched diets were administered twice daily (morning 0900-1000h; evening 1600-1700h) to satiation (≈5% body

weight per meal). Once the E2 treatments ceased, the juveniles were slowly weaned onto commercial micropellets (Hikari®, Japan) over a 10 day period. The survival rates of the juveniles were recorded at 30 DAP. Observations of the anal fin morphology (Angus et al., 2005) were undertaken at 50 DAP for sex identification.

4.3.4. Statistical Analysis

IBM SPSS Statistical analysis software (version 22) was used to analyse all the data in both experiments. The data was tested for normality (Shapiro-Wilk normality test) prior to Welch's ANOVA or One-way ANOVA analysis followed by Tukey-Kramer or Tukey post-hoc test (where applicable) to determine the differences in gestation period and survival rates between the treatments. The differences between the number of progeny produced in the first and second parturition was analysed with the Paired-samples t-test while the Chi-square (χ^2) test was used to analyse the sex ratio of the treatments and control groups (Quinn and Keough, 2002). Differences were considered to be significant at $P<0.05$. All data are presented as mean ± standard deviation unless otherwise stated.

4.4. Results

4.4.1. Experiment 1: Gestation period, clutch size and gonad morphology of gravid females treated with E2

Data on the parturition, gestation period, treatment period pre-parturition, total treatment period and number of progeny produced by gravid *G. holbrooki* females before and after E2 treatments are compiled in Table 4.1. Out of 35 gravid *G. holbrooki* females, only one female did not parturate at the end of the 30 days experimental period. Mean gestation period observed in this experiment was 29.28 ± 3.1 days. The longest gestation period observed was 35 days with 24 days being the shortest. No significant difference ($P>0.05$) was found in the gestation period between the individuals assigned to the treatments and control groups.

On initiation of the treatment there was a significant decrease in the number of progeny produced between the first and second parturition at the respective control and treatment groups. The mean number of progeny produced in the first parturition event was 53 ±30.5 while the mean produced in the second parturition was 23 ±11.5, an average decrease of 20 ± 27.5 progeny ((95% CI, 20 to 39), $t(33)=6.315$, $P<0.05$). The embryos were exposed to E2 for 10- 21 days depending on the gestation period of the female.

Table 4.1. Number progeny, gestation period and exposure period of female *G. holbrooki* treated with E2

Treatment	Female	1st Parturition* (#progeny)	Gestation Period (days)	2nd Parturition (#progeny)	Embryo exposure period (days)
C1 (without Ethanol)	F1	25	27	18	-
	F2	17	28	13	-
	F3	128	27	55	-
	F4	73	27	28	-
	F5	29	26	41	-
C2 (with 70% Ethanol)	F1	98	32	17	18
	F2	36	34	14	20
	F3	31	35	18	21
	F4	91	28	53	14
	F5	86	30	21	16
T1 50 mg/kg feed	F1	88	29	13	15
	F2+	19	-	-	-
	F3	56	24	29	10
	F4	26	30	25	16
	F5	48	35	20	21
T2 100 mg/kg feed	F1	17	34	29	20
	F2	29	32	13	18
	F3	127	27	24	13
	F4	26	34	22	20
	F5	45	35	20	21
T3 200 mg/kg feed	F1	44	35	11	21
	F2	62	27	24	13
	F3	61	26	21	12
	F4	46	30	17	16
	F5	24	33	15	19
T4 300 mg/kg feed	F1	50	31	21	17
	F2	37	33	7	19
	F3	14	31	19	17
	F4	44	33	8	19
	F5	80	30	43	16
T5 400 mg/kg feed	F1	86	29	30	15
	F2	56	26	20	12
	F3	25	29	36	15
	F4	75	28	33	14
	F5	29	30	18	16

*juveniles born prior to hormone treatment that were used in Experiment 2; + second parturition did not occur.

When examined at the termination of treatment, the gonads of females in both control and treatment groups appeared to be “normal” with few bearing embryos at the third and fourth stage while some only contained unfertilized mature eggs (Fig. 4.2; Table 4.2), but no signs of atrophy were apparent.

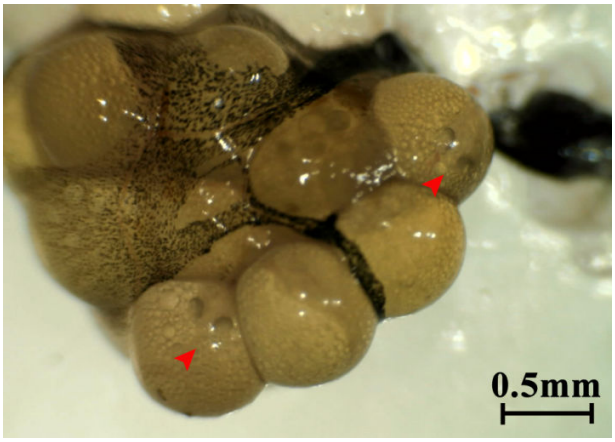


Fig. 4.2. Gonad of *G. holbrooki* females at the end of the E2 treatment period. Embryos at the second developmental stage are shown by red arrow.

Table 4.2. Gonad content of controls and treated females at the end of E2 treatment (30 days).

Treatment	Females	Gonad Content		
		Mature unfertilized Eggs	Third Stage Embryo	Fourth Stage Embryo
C1 (without Ethanol)	F1	✓	✓	
	F2	✓		
	F3	✓	✓	✓
	F4	✓	✓	✓
	F5	✓		
C2 (with 70% Ethanol)	F1	✓		✓
	F2	✓	✓	
	F3	✓	✓	✓
	F4	✓	✓	
	F5	✓	✓	
T1 - 20 mg/kg feed	F1	✓		
	F2	✓	✓	
	F3	✓		
	F4	✓	✓	✓
	F5	✓		✓
T2 - 40 mg/kg feed	F1	✓	✓	
	F2	✓	✓	✓
	F3	✓		✓
	F4	✓	✓	
	F5	✓		
T3 - 60 mg/kg feed	F1	✓		
	F2	✓	✓	✓
	F3	✓	✓	
	F4	✓	✓	
	F5	✓	✓	
T4 - 80 mg/kg feed	F1	✓	✓	
	F2	✓		
	F3	✓	✓	
	F4	✓		
	F5	✓	✓	✓
T5 - 100 mg/kg feed	F1	✓		
	F2	✓	✓	✓
	F3	✓	✓	
	F4	✓	✓	
	F5	✓	✓	

4.4.2. Experiment 1: Effects of E2 on survival rates of *G. holbrooki* exposed during the embryonic developmental stage

The highest MSR of juveniles was observed in C1 at 79.96±20.327% and this was followed by C2 at 77.09±10.32% (Fig. 4.3). The highest juvenile MSR among treatment groups was observed in T1 (61.64±9.34%) followed by T2 (59.33±12.54%), T3 (57.51±9.3%) and T4 (54.95±5.58%) respectively. Finally, the MSR was 51.59±6.04 % for juveniles exposed to the highest E2 concentration (T5). One-way ANOVA analysis found a significant difference ($F=4.38$; df: 6, 27; $P<0.05$) in the juvenile MSR of all the groups including controls although Tukey-Kramer post hoc test shows that there were no significant differences between controls (C1 and C2) and T1-T3; C2 and T1-T4 as well as between T1-T5 .

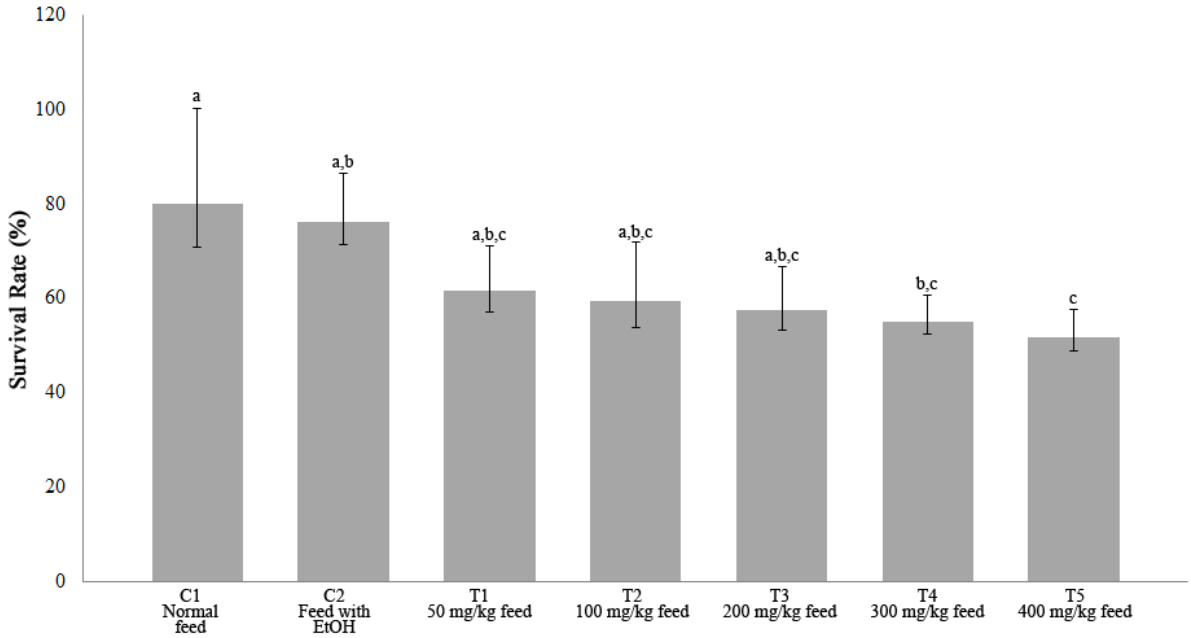


Fig. 4.3. Survival rate (mean±SD) of juveniles treated with E2 during the embryonic life stage at 30DAP. Groups with the same superscript were not significantly different from each other ($P>0.05$).

4.4.3. Experiment 2: Effect of E2 on survival rates of *G. holbrooki* exposed during the juvenile stage

The MSR of juveniles in both control groups, C1 and C2 were 71.73±22.86% and 70.02±18.26% respectively (Fig. 4.4). The highest juvenile MSR among the E2 exposed fish was displayed by T1 at 66.38±12.34% followed by juveniles of T2 at 59.83±11.09 %; T3 at 34.86 ±28.31% and T4 with only 15.19±15.27%. No juveniles survived the highest E2 concentration (T5: 400 mg/kg feed) by the end of the treatment period. Statistical analysis showed that there was a significant difference in the MSR of juveniles between all the controls and treatment groups ($F=7.27$; df: 5, 24; $P<0.05$). Further analysis using Tukey post hoc test showed that there was no significant differences between controls and T1-T3 as well as between T3 and T4.

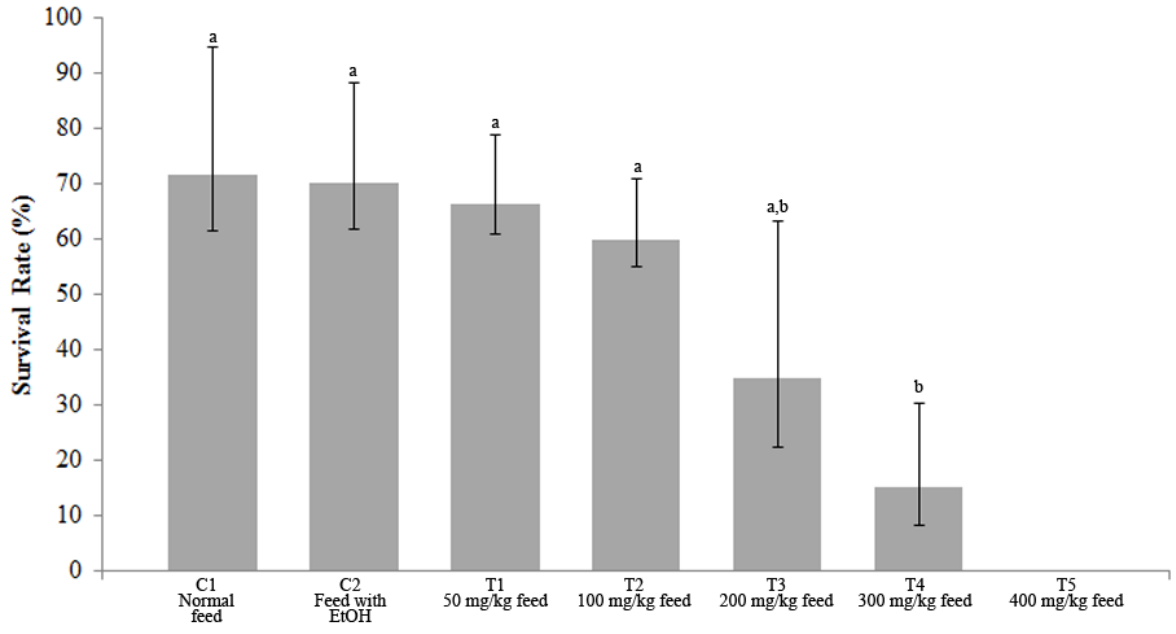


Fig. 4.4. Survival rate (mean±SD) of juveniles exposed to E2 at the end of treatment period. Groups with the same superscript were not significantly different from each other ($P>0.05$).

4.4.4. Effects of E2 on sex ratio of *G. holbrooki*

In experiment 1, females treated with E2 dosages of 200-400 mg/kg produced a 100% female population (Table 4.2). For the two lowest E2 concentrations, T1 and T2, although an all-female population was not produced, the Chi-square analysis showed that the sex ratio of *G. holbrooki* in both groups significantly deviated from the 1:1 male:female ratio. In experiment 2, a 100% female population was achieved in all the treatment groups. In contrast, the sex ratios of the control groups in both experiments were not significantly different from the expected 1:1 male:female sex ratio.

Table 4.2. Percentage of sex ratio in control and treatment groups of both experiments at 50 DAP

Experiment	Treatments	Sex Ratio (%)		χ^2	df	P
		Male	Female			
Experiment 1	C1	56.5	43.5	0.360	1	>0.05
	C2	56.3	43.7	0.640	1	>0.05
	T1	39.9	60.1	4.000	1	<0.05
	T2	14.2	85.8	51.840	1	<0.05
	T3	-	100			
	T4	-	100			
	T5	-	100			
Experiment 2	C1	54.5	45.5	0.802	1	>0.05
	C2	47.7	52.3	0.160	1	>0.05
	T1	-	100			
	T2	-	100			
	T3	-	100			
	T4	-	100			
	T5	-	100			

4.5. Discussion

The aim of this study was to determine the efficacy of estradiol (E2) on the sex reversal of *G. holbrooki* with a view to develop protocols for feminizing this species as a step in the TSC strategy. In contrast, most studies on the effects of sex steroids in *G. holbrooki* have been undertaken in the context of investigating the effects of endocrine disrupting compounds (EDC), primarily using secondary sexual characteristics as markers (for example, Brockmeier et al. (2013), Midgley et al. (2014)). The findings of the present study show that E2 is a suitable hormone to promote feminization in *G. holbrooki*. Here, the result of the E2 exposure treatment in two different life stages of *G. holbrooki* is discussed.

4.5.1. Short term effects of E2 on gravid females

Compared to other teleosts, the technique of sex reversal in livebearers is quite unique, particularly when embryonic stages are targeted for treatment. In egg-laying species, the eggs/embryos can be administered with desired hormones directly via the immersion method (Piferrer and Donaldson, 1994) whereas in livebearers, it was suggested that indirect administration through gravid females is necessary (Pandian and Sheela, 1995), thus affecting the endocrine pathways of the gravid females. Our observation revealed that there was no apparent effect on the reproductive output of gravid *G. holbrooki* females after 30 days of exposure to E2. Although there was a significant decrease in the clutch size between the first and second parturition, it was unlikely that this was caused by E2 interference with fertilisation as internal fertilization had occurred before the hormone was administered. The occurrence of internal fertilization before treatment begun was confirmed based on the development and

appearance of the gravid spot (the gravid spot becomes bigger and darker with the progression of embryos as described in Norazmi-Lokman et al. (2016)). Furthermore, our routine observation on unexposed *G. holbrooki* females also displayed the same pattern of clutch size reduction in subsequent parturitions. The cause for reduction in clutch size in subsequent parturition remains unknown. The mean gestation period observed in this study, 29.28 ± 3.1 was comparable to the 28 days gestation period that was routinely observed in our laboratory (Norazmi-Lokman et al., 2016) and was not significantly different to the respective controls. 'This study suggests that gestation period was not affected by E2 treatment. It has also been suggested that E2 does not play any role during embryonic growth and development except for sex differentiation (Nakamura et al., 2003). Nonetheless, a higher E2 concentration compared to those used in this study might affect the gestation period and clutch size due to E2 toxicity.

The observation that the gonad morphology at termination was comparable to those of the control group suggests that the E2 exposure did not significantly perturb the process of re-maturation in the species. E2 treatments also seem to have no effect on the internal fertilization mechanism of this species as developing embryos were observed in the treated females. Nevertheless, it is important to bear in mind that these are only preliminary observations and subtle perturbations at cellular and molecular levels cannot be categorically ruled out. A more detailed study exploring effects of more long term exposure at cellular, molecular, organismal and population levels are necessary. Knowledge on the effects of hormone treatment on reproduction of gravid female livebearers is still very limited. Previous studies on sex reversal of guppies have not reported adverse effects on treated gravid females (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b). Although a few studies have been completed on the

expression levels of aromatase gene in livebearers exposed to EDCs, none have been conducted on gravid females treated with estrogen (Orlando et al., 2002, Hallgren and Olsén, 2010, Brockmeier et al., 2013). Therefore, more detailed and specific studies on the effects of hormone treatment of gravid females in this species are warranted.

4.5.2. Survival rates of E2 treated fish

Overall, *G. holbrooki* treated during the embryonic developmental stage showed a better resilience to E2 treatment compared to newly born juveniles as shown by the MSRs. This might be attributed to factors that may have caused a difference in the level of E2 uptake in the two experiments. The actual quantities of E2 delivered to the treated fish are shown in Appendix 4. Although hormone exposure appears higher in gravid females, the quantity of E2 delivered to the embryos is unknown since most of it would be absorbed or metabolised by the mother, requiring higher hormone dose to sex reverse poecilids compared to other teleosts (Piferrer, 2001).. The better survival rates of the juveniles in Experiment 1 could be attributed to a more regulated delivery of hormones to the embryos plus shorter duration of E2 exposure (12-21 days). In contrast, the juveniles in Experiment 2 were exposed to relatively unregulated and perhaps higher levels of E2 than the nominal since the level of E2 ingestion by the juveniles is likely to be different among individuals and their E2 exposure duration is much longer (30 days) compared to embryos in Experiment 1.

The MSR of *G. holbrooki* treated during the embryonic stage in this study are lower compared to those reported in other livebearers. For example, the MSR of guppy produced at E2 concentration of 200 mg/kg was 78.51±10.61% (Kavumpurath and Pandian, 1992) while in the

current study, at the same concentration, the MSR of *G. holbrooki* was only 57.51±9.3%. Similarly juvenile molly treated with E2 at 400 mg/kg had a 65% survival (George and Pandian, 1995) compared to 0% in this study. Nonetheless, a similar pattern of decreased survival with increased E2 was observed in the molly (George and Pandian, 1995). The MSR performance of the present study is much higher compared to the study done on *G. affinis* exposed to E2 via the immersion method. The survival rate of juveniles treated at various E2 concentrations in that study was between 0 to 21% (Senior, 2013).

The variability in survival rates could potentially be attributed to the stocking rates of the juveniles because, in the present study, all the juveniles produced by each female were held together as a batch in a single container without taking into account the number of juveniles in each batch, with the volume of water in each container constant. Our observations also show that the MSR of replicate tanks with lower stocking density is significantly higher compared to those with higher stocking density not only in the E2 treatments but also in the control groups especially in Experiment 2 ($F=21.95$; df: 1,28; $P<0.05$). Fish stocking density plays an important role in determining the performance of survival rate in aquaculture species (El-Sayed, 2002). A few factors that lead to low survival rate at high stocking density are food competition, poor water quality due to crowding and aggressive behaviour among the fish (M'balaka et al., 2012). Since the water quality was maintained within acceptable limits, batch specific aggression might be one of the contributors for low survival rates in the respective batches. Routine visual observations suggest that the aggressiveness of *G. holbrooki* starts as early as 2-3 days after birth where dominant fish attack and nip the fins of weaker fish. It is therefore possible to achieve

higher MSR by reducing the stocking density and/or providing refuges. It is suggested that future studies should consider a stocking density of five fry per litre of rearing water.

4.5.3. E2 can induce total feminization in *G. holbrooki*

Total feminization of *G. holbrooki* was successfully achieved in both experiments. In the first experiment, total feminization was achieved starting at an E2 concentration of 200 mg/kg of feed at the minimum exposure period of 12 days. In guppy, 100% feminization of progeny was achieved at E2 concentration of 400 mg/kg of feed for only 6 days where the treatment started 20 days after the first parturition occurred (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b). There is a likelihood that the longer exposure period in *G. holbrooki* contributed to the lower concentration of E2 required to achieve total feminization compared to guppy.

For the second experiment, 100% feminization was achieved in all E2 treatments. Compared to the present study, a 100% feminization was achieved in molly at the concentration of 200 mg/kg of feed within the same 30 days duration period (George and Pandian, 1995). It is possible that the juveniles of *G. holbrooki* are much more susceptible to E2 treatment compared to molly as is also supported by higher survival rates of molly, reflecting species specific differences.

Comparing the efficiencies of administration routes, it appears that either approach is effective, with some obvious differences. For example, treating at the embryonic stage (via mother) required a much higher dose (200 mg/kg) to achieve 100% feminization but required shorter

treatment duration (minimum 12 days from the starting point of the treatment on gravid females to parturition). In contrast 100% feminization was achieved by treating newborn juveniles even at lowest concentration (50 mg/kg) but with longer treatment duration (30 days). Notably both approaches had comparable MSR. There is a possibility that 100% feminisation in newborn juveniles could be achieved in a shorter treatment duration. Therefore further studies on treatments at lower doses and shorter duration are warranted.

While this study has established reliable protocols for the feminization of *G. holbrooki*, additional progeny testing and development of sex markers are necessary before the Trojan carrier females can be generated for the TSC strategy. Critically, an ability to identify individuals with a male genotype and a female phenotype is necessary. This is important not only for monitoring the progress of TSC, but more immediately for producing the Trojan females (YY females). Although this can be done via progeny testing similar to those reported for guppy and molly, the development of a genetic sex marker will circumvent progeny testing. Progeny testing takes a longer time to conduct and requires a large commitment of facilities (Dunham, 2011), particularly when testing a large number of founders. A genetic sex marker has been developed and effectively used in identifying individuals with opposing genotype and phenotype in *G. affinis* (Senior, 2013, Lamatsch et al., 2015) but not in *G. holbrooki.*

Another aspect that needs addressing is the reproductive fitness of the sex-reversed fish. Based on the analysis by Senior et al. (2012), the reproductive fitness of hormone induced fish are lower compared to normal individuals. Therefore a study needs to be conducted to investigate the potential compromises in reproductive fitness of sex-reversed individuals compared to

controls. This issue is addressed in chapter five of this thesis. There is still room for further improvement and refinement on the feminization protocol of *G. holbrooki* using E2 such as testing it on juveniles at a lower concentration (below 50 mg/kg feed). Nevertheless, the present study has provided significant information that is crucial as a kick-start for the production of Trojan females in *G. holbrooki* to control and eradicate this noxious pest.

4.6 Conclusion

This study has shown that estradiol is effective in sex-reversing the eastern mosquitofish *G. holbrooki*. Specifically, this species can be feminized by E2 treatment during its embryonic life at a concentration of 200 mg/kg of feed for a minimum period of 12 days or during its juvenile stage at a lower E2 concentration of 50 mg/kg of feed but with a longer exposure period of 30 days. The knowledge and protocols developed in this study will assist further refinement of the sex-reversal strategies and deployment of TSC strategy to eradicate this invasive species. Further investigations to develop a genetic sex marker and determination of reproductive fitness of sex reversed individuals are necessary.

CHAPTER 5

Reproductive Fitness of The Eastern Mosquitofish *Gambusia holbrooki* Treated with Estradiol

5.1. Abstract

Evaluating the reproductive fitness of hormone treated individuals and their progeny is critical for improving production efficiencies in the aquaculture sector and for inferring the impacts of endocrine disrupting compound (EDC) pollution in ecotoxicology studies. For pest fish management via the Trojan Sex Chromosome (TSC) strategy, information on the reproductive fitness of hormone treated fish is critical to determine the effectiveness of the TSC strategy in the target species. With this goal, three key reproductive traits of the noxious eastern mosquitofish (G*ambusia holbrooki*) treated with estradiol (E2): (i) ability to breed (ii) gestation period and (iii) clutch size were assessed. Three groups were chosen based on the efficacy of the estradiol treatments (chapter four). The groups were: group one – fish treated with E2 at 200 mg/kg of feed (treatment on embryos via gravid female ingestion); group two – juveniles exposed to E2 concentration of 50 mg/kg of feed (treatment on newborn juveniles) and group three – controls (unexposed fish). Almost all females were gravid and successfully parturated where the average percentage was 71.5% for group one, 73.0% for group two and 72.23% for group three, of which less than half (43.7% females in group one, 40.5% in group two and 45.0% in group three) of the females managed to parturate for the second time. There was no significant difference in the percentages of females that parturated between exposed and control groups during the first and second parturition event ($P>0.05$). The mean gestation period observed (28.98 ± 3.3, 28.2 ± 2.36 and 29.8 ± 3.17 days in groups one, two and three respectively) was not significantly different between the three groups. The average clutch size in all the three groups was relatively small compared to previous observations (chapter 2-4) but there was no significant difference between the groups at both first and second parturition events. Each group produced an average of two to four progenies. The mean survival rates (MSR) of the progenies produced by females of all three

groups were high. No significant difference was found in the MSR of progenies between the three groups during the first (100%, 90 ± 20%, 92 ± 16% in groups one, two and three respectively) and second (Group one: 95 ± 10 %, group two: 95 ± 10% and group three: 100%) parturition event. Overall, this study demonstrates that the reproductive fitness of E2 treated fish is on par with the control. Reasons for small clutch size in both treated and control groups are as yet unclear. However, noting that all females in these observations were brooding for the first time, age and size of the brood fish may have a role. A long-term observation and further studies are therefore necessary.

Keywords: Gambusia, Invasive fish, Live bearing fish, Endocrine Disrupting Compound, Breeding, Clutch Size

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5.2. Introduction

Fish are deliberately and inadvertently exposed to sex steroids. For example, in the aquaculture sector, EDCs such as estradiol (E2) or diethylstilbestrol (DES) are deliberately administered to fish to generate monosex progenies with a view to increase production and profits (Pandian and Sheela, 1995, Piferrer, 2001, Devlin and Nagahama, 2002, Cnaani and Levavi-Sivan, 2009). Meanwhile, aquatic pollution due to domestic and commercial effluent which contain hormones or hormone-mimic compounds are the main cause of inadvertent exposure of EDC on fish in the environment (Álvarez-Muñoz et al., 2015, Barber et al., 2015). In either case indiscriminate exposure is known to cause adverse side effects on organisms exposed to EDC. Several body systems and functions such as development, reproduction and behaviour are affected by EDC due to its ability to interfere and disrupt various functions such as the synthesis or secretion of endogenous hormones, in the endocrine system (Segner et al., 2003).

The impact of EDC on fish reproductive fitness has been a matter of concern since it has a direct effect on sustainability and livelihoods of both cultured and wild fisheries. In the aquaculture sector, the success of hormone sex reversal is also determined by the reproductive fitness of exposed fish since it determines the ability to propagate and raise fish seed necessary for large scale farming operations (Pandian and Sheela, 1995, Piferrer, 2001) whilst in ecotoxicology studies, information on the effects of EDC on fish reproduction provides valuable insights into aquatic contamination thus acting as a bioindicator of aquatic pollution (Allner et al., 2010).

More recently, hormone sex reversal has been suggested as a key technique to develop a genetic option for pest fish control as part of the Trojan Sex Chromosome (TSC) strategy (Gutierrez and

Teem, 2006, Cotton and Wedekind, 2007, Stelkens and Wedekind, 2010, Schill et al., 2016). Although TSC is currently not employed in any species, various models have been developed to demonstrate its efficiency as a potential bio-control for pest fish (for example; Cotton and Wedekind (2007), Stelkens and Wedekind (2010), Wang et al. (2016)). However, all the models assume that the Trojan chromosome carriers (produced via hormone sex reversal) have the same reproductive fitness as normal fish. For example, Trojan fish are assumed to produce the same number of progeny as normal fish. Based on a meta-analysis conducted on various literature related to fish hormone sex reversal, the reproductive fitness of sex reversed fish is lower than normal fish thus there are possibilities that the Trojan fish reproductive fitness is compromised (Senior et al., 2012). Therefore, information on the impacts of EDC on the reproductive fitness of sex reversed fish is crucial to evaluate the effectiveness of the TSC strategy in the target species (Senior et al., 2012, Senior et al., 2013).

The reproductive abilities of fully sex reversed fish produced using E2 have been reported in other species such as guppy (*Poecilia reticulata*), black molly (*P. sphenops*), golden rosy barb (*Puntius conchonius*), bluegill sunfish (*Lepomis macrochirus*), yellow catfish (*Pelteobagrus fulvidraco*), and brook trout (*Salvelinus fontinalis*) (Kavumpurath and Pandian, 1993b, George and Pandian, 1995, Kirankumar et al., 2003, Wang et al., 2008, Liu et al., 2012, Schill et al., 2016). However, only a few of these studies discuss the ability of the sex reversed fish to breed based on progeny testing experiments, gonad histological studies or production of YY individuals. Importantly, more critical aspects of reproduction such as fecundity or gestation of E2 exposed fish remain poorly documented especially in livebearers. In *G. holbrooki,* the effects of E2 on reproduction have been reported on exposed males (Doyle and Lim, 2002, Doyle and

Lim, 2005) but not in fully sex reversed females thus an assessment of the reproductive fitness of these females is warranted.

As a first step towards developing a TSC strategy for control of the eastern mosquitofish, *Gambusia holbrooki*, E2 treatment has been shown to produce complete sex-reversal (chapter four). Nonetheless, the reproductive fitness of the sex reversed individuals in this species is yet to be assessed. Therefore, this study was set to systematically assess the effects of E2 on the following reproductive traits: breeding ability; gestation period and clutch size of exposed *G. holbrooki*

5.3. Materials and methods

5.3.1. Source of specimens

Estradiol treated *G. holbrooki* produced in the estradiol (E2) treatment study (chapter four) were reared to maturity. Females were considered mature as soon as they possessed the gravid spot (Figure 5.1) (Kristensen et al., 2007). Fish that survived to maturation (18.0 – 28.0 mm TL) from the treatment with E2 at the concentration of 200 mg/kg from the first experiment (exposed during embryonic stage) and 50 mg/kg from the second experiment (exposed newborn juveniles) along with control groups were used in this study. Males (16.0 – 25.0 mm TL) used in this study were obtained from controls and hatchery stock (routine breeding). These males were separated from the females as soon as the third anal fin ray thickens at 50 days after parturition (DAP) (prior to maturation) (Angus et al., 2005) to ensure that they were virgin to avoid any bias during breeding activity. They were used for mating once the gonopodium was fully developed.

Fig. 5.1. Mature *G. holbrooki* exposed to E2 during the embryonic development stage. The gravid spot is shown by the red arrow (scale in cm).

5.3.2. Fish rearing and breeding

This study was conducted from mid-September 2015 until early January 2016. Throughout this study, all the mature fish (70-90 DAP) were reared in static tanks (5 fish/L, 0ppt, ±25°C, 16L : 8D – lights on at 06.00h) that were each supplied with gentle aeration (Figure 5.2A). Water quality was maintained within acceptable limits by water exchanges which were undertaken every two days using aged and aerated tap water. The fish were divided into 3 groups; Group one – fish that were treated with E2 at 200 mg/kg feed (from the first experiment of chapter four; n=42); Group two – those exposed to E2 concentration of 50 mg/kg feed (second experiment of chapter four; n=45) and Group three – controls (unexposed fish; n=47 from Experiment 1 and 2 of chapter four). They were housed based on their respective clutches (five tanks each group) with each tank containing three to 16 females. The water volume in the tanks was adjusted to maintain the stocking density (5 fish/L). Commercial micropellets (Hikari®, Japan) were delivered by hand to the fish twice a day to satiation (≈5% body weight per meal; morning 0900-1000h; evening 1600-1700h).

Virgin males were introduced at the beginning of the experiment where the sex ratio in each tank was set at 1 : 2 (male : female) following the sex ratio used in guppy breeding (Rikowskiy, 2015) (See Supplementary Video 2). Any dead males were immediately replaced with new males. As soon as the females showed signs of gravidity (intense gravid spot), each female was transferred to a separate static tank fitted with a breeding trap (Figure 5.2B). Once parturition occurred, the progenies were transferred into a new tank while the female continued to be reared in the same tank until the second parturition occurred. If the second parturition did not occur after 45 days, they were transferred back into the breeding tanks. As fertilization occurs internally, it is difficult

to determine when it actually occurred. Therefore each gestation period was measured as the number of days between two consecutive parturitions (Norazmi-Lokman et al., 2016).

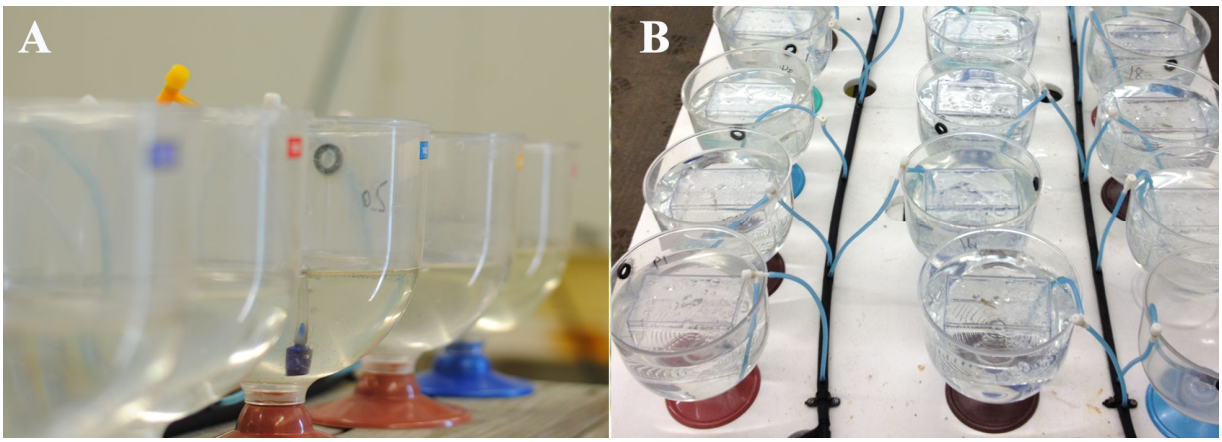

Fig. 5.2. Static tanks set up used in the study. (A) Breeding tanks supplied with gentle aeration. (B) Parturition tanks fitted with breeding traps.

Progenies produced from the breeding were fed with commercial powdered spirulina (Bioglan, Australia) until 30 DAP. They were then slowly weaned onto commercial micro pellets (Hikari®, Japan) over a 10 day period. The survival rate of the progenies were determined at 50 DAP (age where the sex can be determined). Figure 5.3 illustrates the breeding strategy adopted in this study.

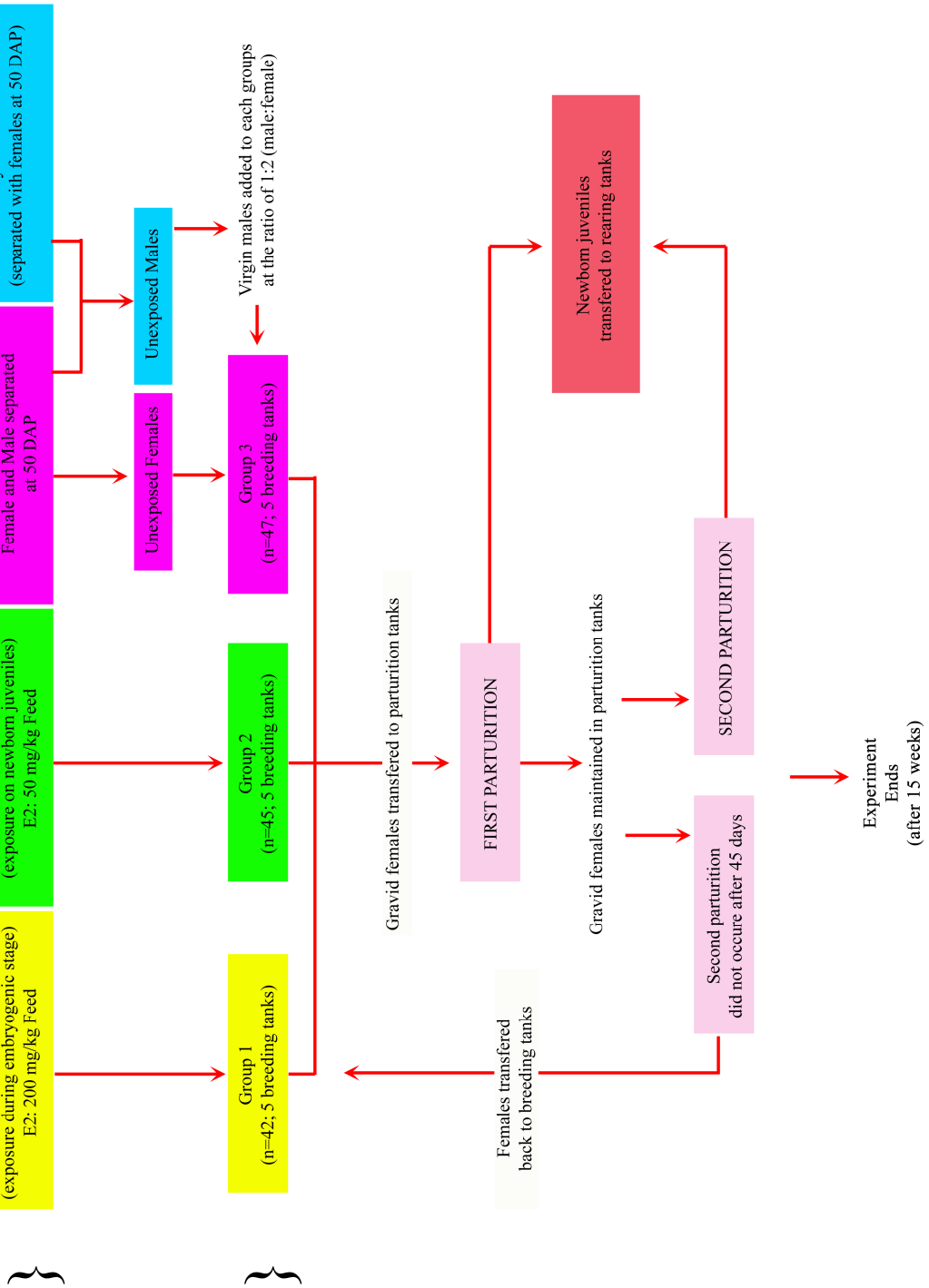

Fig. 5.3. Schematic representation of the breeding strategy used for assessing the reproductive fitness of E2 treated *G. holbrooki*.

5.3.3. Statistical analysis

The data were analysed statistically by using IBM SPSS Statistic software (version 22). One-Way ANOVA was used to determine differences between the three groups in relation to the mean survival rates, gestation period and number of progeny produced after the data was tested for normality (Shapiro-Wilk normality test). Differences were considered to be significant at $P<0.05$ (Quinn and Keough, 2002). All data are presented as mean ± standard deviation unless otherwise stated.

5.4. Results

5.4.1. Gravidity, parturition and gestation period

More than half of the females were gravid and successfully parturated in each group. For group one (exposure during the embryonic stages), an average of 68.67 ±5.72% females were gravid and parturated while it was 71.15 ± 8.83% for group two (exposure during juvenile stage) and 74.14 ± 12.54% for group three (unexposed females)(Fig 5.3). One-way ANOVA analysis confirmed that there was no significant difference between the three groups. Of the females that parturated the first time, less than half managed to parturate a second time. The mean percentage of females parturated for the second time by the end of the study period was 43.89 ± 6.19% females in group one, 40.01 ± 5.87% in group two and 45.83 ± 11.18% in group three (Fig 5.4). There was no significant difference between the percentages of females that managed to parturate for the second time. Across all groups, the shortest gestation period observed in this study was 24 days while the longest was 32 days. The mean gestation period was 28.98 ± 3.3 days for females in group one, 28.2 ± 2.36 days for group two and 29.8 ± 3.17 days for group three. There were no significant differences found in the gestation period between the three groups. Throughout the breeding program, a total of 16 males and 5 females from all three groups died due to aggressive behaviour (see Supplementary Video 3).

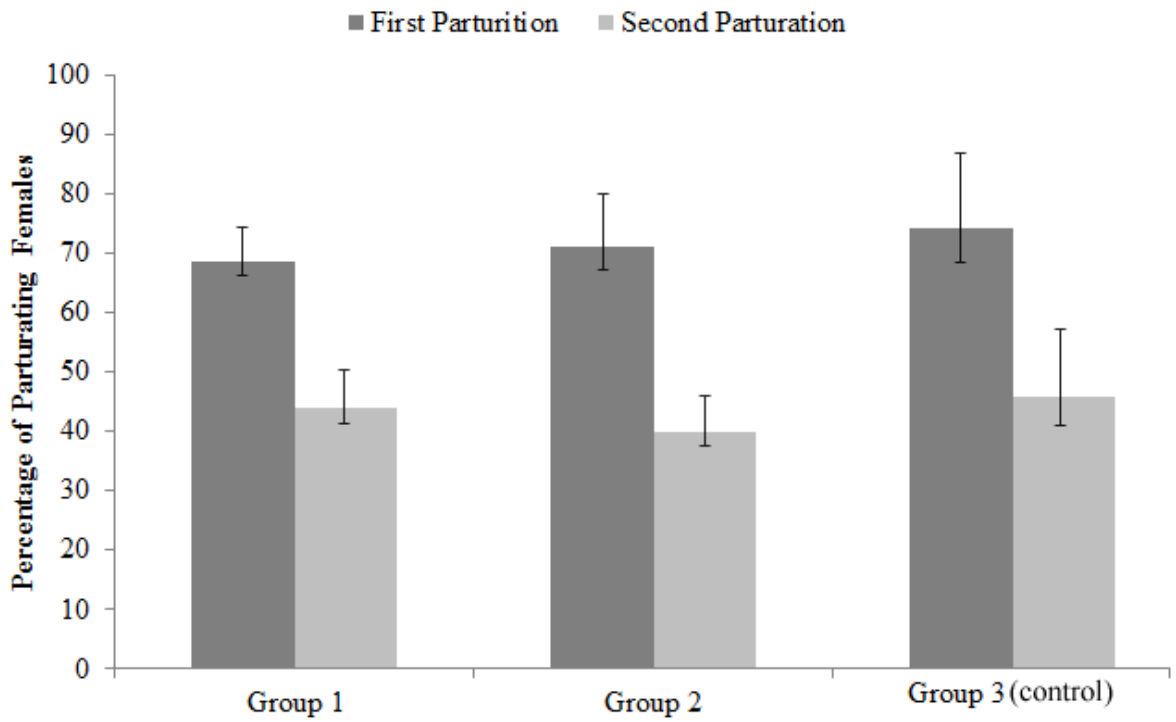

Fig 5.4. Mean percentage of females (±SD) that successfully parturated once (first parturition) and twice (second parturition was a percentage from those that parturated the first time not from the total number of fish used in the study).

5.4.2. Clutch size and survival rates

The number of progeny produced by females in all three groups ranged from 1-5 in each of the two parturition events (Fig 5.5). The average number of progenies produced during the first and second parturition were 2.63 ± 1.3 and 2.25 ± 1.08 (Group 1), 3.12 ±1.27 and 3.75 ± 0.96 (Group 2) and 2.75 ±1.64 and 2.86 ± 1.27 (Group 3) progenies. There were no significant differences in the average number of progenies produced by all three groups during the first and second parturition.

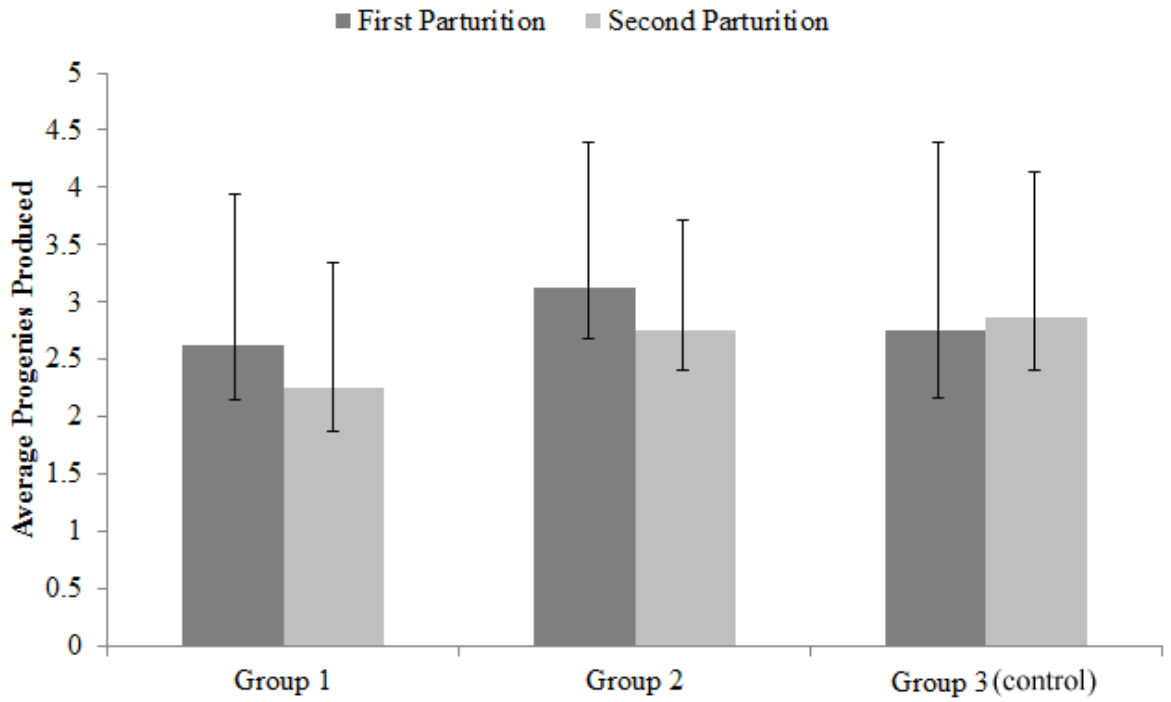

Fig 5.5. Average number (± SD) of progenies produced during the first and second parturition.

A high mean survival rate (MSR) was observed in all fish groups. In the first parturition event, the MSR was 100% for progenies in group 1, 90 ± 20% (±SD) in group 2 and 92 ± 16% in group 3 (Fig. 5.6). For the second parturition event, the MSR of progenies in group one and two were both 95 ± 10% while it was 100% for progenies in group 3 (Fig. 5.6). No significant difference was found in the MSR between the progenies from the three groups during both parturition events.

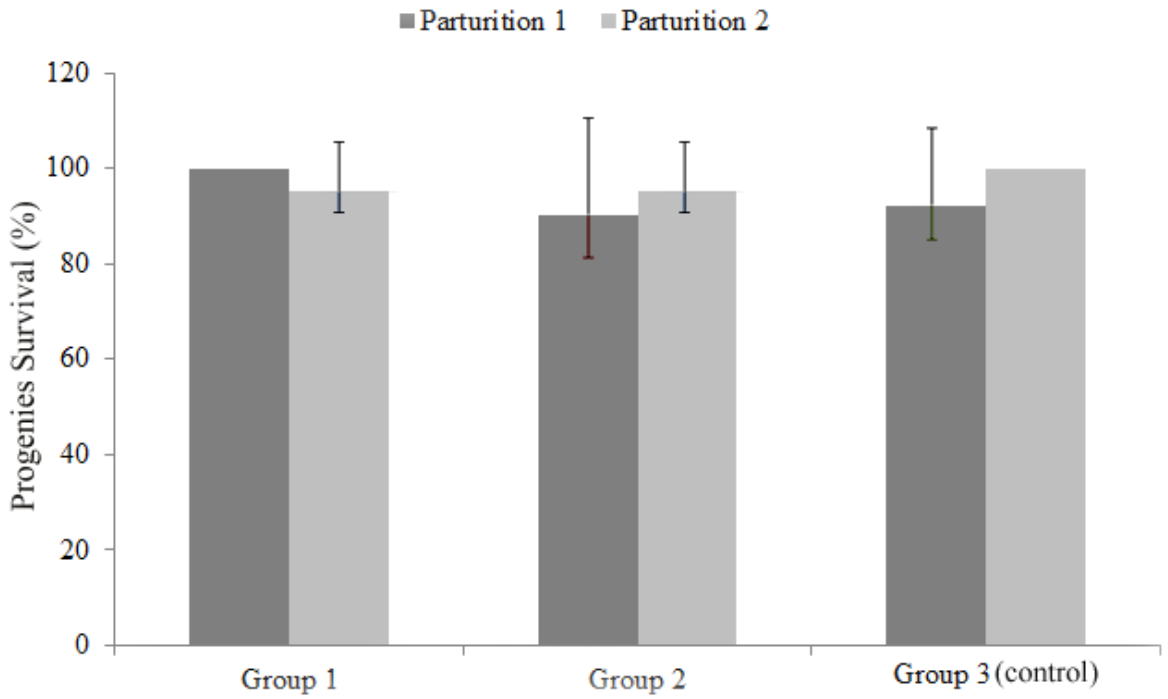

Fig 5.6. Mean survival rates (MSR) percentage (±SD) of progenies in the first and second parturition events.

5.5. Discussion

The main aim of this study was to assess the reproductive fitness of the fish generated following E2 treatment (chapter four). The reproductive fitness of the treated fish was assessed against controls to determine whether hormone sex reversal affected reproductive ability and performance. The study shows that there were no significant effects of E2 on the reproductive fitness of the treated fish in terms of reproductive abilities and gestation period. Here the findings of this study are discussed and compared.

5.5.1. Estradiol treatment did not affect *G. holbrooki* reproduction

Almost all the females in the three groups were gravid and successfully parturated, suggesting that the exposure to E2 did not affect the mating and gravidity of *G. holbrooki* in this study. The ability of E2 treated *G. holbrooki* to breed is crucial in the production of Trojan females for the TSC strategy. Production of Trojan females involves the breeding of feminised sex reversed fish, in the case of *G. holbrooki*, a female fish with XY chromosome (also known as neo female) where the progenies produced will be sex reversed again to produce YY females (the Trojan fish) (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007). Furthermore, these hormone treated YY females must have the ability to breed with normal males and have the same reproductive fitness with normal females in order for the TSC to be effective (Senior et al., 2012, Senior et al., 2013).

Studies on the sex reversal using E2 treatments in other species have reported a similar ability of hormone exposed individuals to breed, but lack comparative assessment of fitness. For example,

in guppy (*Poecilia reticulata*), a species that belongs in the same family as *G. holbrooki*, progeny testing was undertaken on randomly selected treated females without detailed comparison with control females (Kavumpurath and Pandian, 1993b). This was also the case in the reports on black molly (*P. sphenops*) and golden rosy barb (*Puntius conchonius*) (George and Pandian, 1995, Kirankumar et al., 2003). In the bluegill sunfish (*Lepomis macrochirus*), assumption on the ability of E2 sex reversed fish to reproduce was made by comparing histological slides of the gonad of treated and control fish (Wang et al., 2008). The histological studies showed that the sex reversed bluegill sunfish possessed a similar functioning ovary as normal females hence suggesting that the sex reversed bluegill sunfish can be bred. Meanwhile, the ability of yellow catfish (*Pelteobagrus fulvidraco*) and brook trout (*Salvelinus fontinalis*) E2 treated fish to reproduce in their respective studies was confirmed by the production of YY males (Liu et al., 2012, Schill et al., 2016). Nevertheless, it is apparent that E2 exposed fish used in this study have the ability to breed as normal females.

Administration of E2 did not impede the ability to breed (mating) and reproduce in *G. holbrooki* since there were no statistical differences in the number of exposed fish parturated compared to controls. However there is a concern about the effect of interactions between fish which may disturb breeding. A behavioural study on *G. affinis* has shown that control females displayed aggressive behaviour towards sex reversed females (Senior, 2013) potentially disturbing mating of sex reversed females. In the current study, breeding of the E2 exposed *G. holbrooki* and controls was performed separately, each in their own group (control and hormone exposed fish were not housed in the same tank). There is some likelihood that the number of E2 exposed

gravid females will be reduced due to aggression and mating competition if they were housed together with the controls.

It is unknown why the remainder of the females in the three groups did not parturate although they possessed a gravid spot (which clearly show that they were mature). There are a number of possibilities: they were actually gravid and parturition in the breeding tank went unnoticed and the progenies were eaten by larger fish in the tank, or they did not manage to mate with males due to aggression by larger females (as seen in the third supplementary video) in the group since large females tends to dominate (Deaton, 2008a, Deaton, 2008b). Analysis on fish size (Appendix 5) shows that the size differences in each clutch is due to natural variation since no significant difference was found between the size of the E2 treated fish and unexposed females. Aggressive behaviour of females observed during mating in this study also caused the death of several females and males. Observations made on the carcasses of the dead fish showed fin nipping occurred especially at the gonopodium in male fish and caudal fin in both sexes. However the number of dead fish was low in all three groups. Chasing behaviour, coercive copulation by males, male mating competition and female avoidance behaviour are common in Gambusia but none of the previous reports have shown that this competitive mating behaviour led to death (Bisazza et al., 2001, Pilastro et al., 2003, Smith, 2007, Deaton, 2008a, Senior, 2013).

At the end of the observation period, about 40% of females in each group managed to parturate for the second time without mating with males for a second time. This confirms the ability of this species to store sperm and use it to fertilise multiple batches of eggs as described in previous

studies (Jobling, 1995, Koya et al., 2000, Bone and Moore, 2007). There were no differences in the percentage of females that managed to parturate for the second time between the exposed fish and controls thus demonstrating that it was unlikely E2 exposure has any long term effects on the occurrence of parturition and internal fertilization in this species. In this study, females that did not parturate for the second time might have expended all the sperm to produce the first clutch of the progeny. The mechanism of sperm storage and internal fertilization in this species is still not fully understood. Routine observation on parturition activities conducted in our laboratory showed that some females can parturate up to four times without mating (unpublished). There were also cases where the female only parturated once and parturated again after mating occurred (unpublished). At the time of writing this chapter, a third parturition had not occurred in any fish group. It might be possible that parturition in virgin females can occur twice without subsequent mating whilst older 'experienced' females are capable of storing sperm to fertilise more than two batches.

The gestation period of exposed females was also not affected since there were no differences between treatment females and control females (28-29 days). The gestation period observed in this study is in agreement with the gestation period observed earlier in unexposed *G. holbrooki* females which is 28 days at 25°C (Norazmi-Lokman et al., 2016). The same observation was also made in the gestation period of black molly where the gestation period of E2 exposed fish was 40 days while control was 39 days (George and Pandian, 1995, George and Pandian, 1998). This strongly suggests that E2 exposure at the optimum dose does not affect the embryonic development of both poecilids.

5.5.2. Effects of E2 on clutch size

The number of progeny produced between the three groups in this study (1-5 progenies) can be considered low. No reports are available on the clutch size produced by 'first-time mothers' in *G. holbrooki* that can be compared. This is also the first time the production of progenies from first time mothers was recorded in our laboratory. Generally parturition and production of progenies employed larger wild caught females that had potentially undergone multiple pregnancies. One of the main reasons for small clutch sizes produced by females in this study is the small size of the females. Size is known to correlate with the fecundity and clutch size in this species where bigger fish produce bigger clutch sizes (Norazmi-Lokman et al., 2016). Unfortunately, it was not possible to apply the equation produced in chapter 2 (to predict clutch size) here since the gravid spot size and intensity was not measured to avoid stress to the fish. The number of progenies produced by 'first-time mothers' in other poecilids is much higher compared to *G. holbrooki* observed in this study. In guppies, the number of progenies reported was 14-34 while it was 9-17 in black molly (Kavumpurath and Pandian, 1993b, George and Pandian, 1995).

Even though there was no significant difference in the number of progenies produced between the three groups, it is too early to assume that E2 does not affect the clutch size and fecundity of *G. holbrooki* since the number of progenies is too small to detect variability. A few reports have shown that E2 treated fish have a lower clutch size and fecundity compared to normal unexposed females in black molly, golden rosy barb, Siamese fighting fish (*Betta splendens*) (Kavumpurath and Pandian, 1993a, George and Pandian, 1995, Kirankumar et al., 2003). Analysis by Senior et al. (2012) found that the gonado-somatic index of fish exposed to EDC is lower than controls

thus suggesting that the fecundity might be reduced. In contrast, the fecundity of brook trout treated with E2 is much higher compared to unexposed fish (Schill et al., 2016).

Continued breeding and observations are therefore necessary to determine if size and age have a significant influence on the size of the clutch. Notably, the MSR observed in all the fish groups was much higher compared to the MSR observed earlier in this species (chapter three and four). This may be attributed to the low stocking density in this study compared to those presented in earlier chapters.

5.5.3. Overview on the effects of EDC exposure on fish reproduction

The findings of this study show that E2 has no impact on *G. holbrooki* reproduction, although continued observations are warranted. In contrast, a number of studies have reported the adverse effect EDC have on fish reproduction both in aquaculture and ecological contexts. There are a few factors that contribute to the compromised reproductive fitness in fish exposed to E2 or any other EDC. The dose/level of EDC and, timing and period/length of exposure play a significant role in influencing the reproduction of exposed fish.

A fully functional sex reversed fish (complete sex reversal) is produced when fish is exposed to an optimal dose of estrogen or androgen during the labile period (Pandian and Sheela, 1995, Piferrer, 2001). Incomplete sex reversal due to insufficient or an overdose of EDC can lead to the development of intersex fish (Hunter and Donaldson, 1983) and intersex gonad and sterility (Yamazaki, 1983) respectively in exposed fish since treatment response is dose-dependent.

Adverse effects of EDC on the reproduction of fish exposed via environmental pollution have been reported and reviewed in many fish species (Nash et al., 2004, Scholz and Klüver, 2009, Harris et al., 2011, Álvarez-Muñoz et al., 2015, Barber et al., 2015, Meijide et al., 2016). EDCs act by interfering with multiple functions in the endocrine system such as the synthesis, secretion, transport, binding and action of endogenous hormones (Segner et al., 2003). Exposure of fish to EDC outside the labile period and for prolonged duration at uncertain dose/level will disrupt the reproduction system. This has been the case for the majority of fish exposed to EDC in the environment. For example, in adult and juvenile *G. holbrooki* males, exposure to waterborne E2 for 84 days outside the labile period (where sex determination has occurred) significantly reduced the reproductive abilities of the males since it interfered with the development of secondary sexual characteristics in the juveniles and lowered the level of sexual activity in both juvenile and adult males (Doyle and Lim, 2002, Doyle and Lim, 2005). In this study, no obvious impacts on *G. holbrooki* reproduction were observed. Therefore the administered E2 dose and duration are optimal or close to optimal.

5.6. Conclusions

Although the effect of E2 on the clutch size of *G. holbrooki* needs further verification, it is clear that E2 has a low impact on the reproductive fitness of this species. Fish exposed to E2 and showing the best performance (highest MSR and feminization percentage) from a previous experiment (chapter four) could be successfully bred, opening a path towards the production of Trojan fish for the TSC strategy to control and eradicate *G. holbrooki*. Focus can now be directed to further observations of the effects of E2 on clutch size to determine the efficiency of TSC as a bio-control for this species. Comparative investigations on the behaviour of sex reversed and normal fish is highly recommended for any similar efforts to develop pest control strategies. Data collected in this study will also serve as a reference for environmental studies investigating the impacts of EDC exposure in wild fish populations.

CHAPTER 6

General Discussion

Manipulation of sex in teleosts via hormone sex reversal has been demonstrated in an array of commercially important fish species such as tilapia and salmonids (Pandian and Sheela, 1995, Piferrer, 2001, Pandian and Kirankumar, 2003). In livebearers, studies on hormone sex reversal have been mainly focused on guppies (*Poecilia reticulata*) (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b), black molly (*P. sphenops*) (George and Pandian, 1995) and swordtails (*Xiphophorus helleri*) (Ortega-Salas et al., 2013) due to their significance in the ornamental fish industry. This study is the first comprehensive investigation on the sex reversal of the eastern mosquitofish, *Gambusia holbrooki*, a pest species in Australia. With the main aim to demonstrate hormonal feminization of *G. holbrooki* for developing the Trojan Sex Chromosome (TSC) approach to control and eradicate this species, this study systematically investigated a number of core aspects that are important to achieve successful hormone sex reversal starting from choosing suitable hormone(s), dose, duration, route and susceptible life stage for treatment including the effects of hormone treatment on fish reproduction.

6.1. Chapter Two – Critical background knowledge

At the beginning of the study, little information was available on hormonal sex reversal in this species, with the exception of sparse literature relating to exposure of endocrine disruption compounds (EDCs). Moreover, most of these studies have been limited to the effects on secondary sex characteristics of sexually differentiated fish (juvenile or mature). The only study conducted on *G. holbrooki* sex reversal was conducted more than 50 years ago (Lepori, 1945 referenced in Piferrer, 2001) and the detailed information on the dosage and protocols is inaccessible. Therefore information such as type and dosage of hormones used was based on those described for other poecilids namely guppy and black molly. Significantly also, key

species specific knowledge as to how to choose suitable candidates/fish for hormone treatment, when to start the treatment on gravid females, and which stage are the developing embryos at the time of treatment (i.e - without sacrificing the female) were also unavailable.

In addition, there was little information available on the artificial propagation of *G. holbrooki*. Whilst this species has been subjected to numerous studies under captive conditions and has been known to be easily maintained in the laboratory (Pyke, 2005), details on its propagation in captivity such as suitable rearing temperature have never been published. This is likely because *G. holbrooki* has always been considered as a species with no economic importance and has never been mass cultured unlike other livebearers such as guppy and swordtails which are popular among aquarium enthusiasts. Nonetheless all this information is important in the development of Trojan female fish in the Trojan Sex Chromosome (TSC) strategy since the process involves the rearing and propagation of multiple batches of *G. holbrooki* under captive conditions (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007). Therefore chapter two of this study focused to address these basic questions even before hormone treatment could be attempted (chapter three and four).

6.1.1. Utility of the gravid spot as a surrogate to predict and assess reproductive outputs.

While the gravid spot has been known to be a sign of maturity in female Gambusia (Kristensen et al., 2007) and is somewhat related to its reproductive cycle (Howell et al., 1980), the relationship between the gravid spot and reproduction in females has not been fully explored and instead focus has been directed to its use in Gambusia copulation behaviour (Deaton, 2008).

Hence chapter two of this study explored and successfully identified the utility of the gravid spot as an excellent marker to predict embryonic development and clutch size in *G. holbrooki* using digital imaging and image analysis techniques. By using information on the intensities obtained from the digital imaging analysis, the embryonic development of *G. holbrooki* can now be determined without sacrificing the brood. An equation to predict clutch size using the relationship between gravid spot intensity and size together with fish length was also ascertained. This information facilitated the sex reversal experiments (chapter three and four) greatly by providing consistency in developmental stages targeted for treatment i.e. assisting in selection of gravid females of similar gravid spot intensity for hormone treatment.

In Gambusia, the labile period for sex reversal has been suggested to occur during the embryonic life stage prior to parturition and just after birth in newborn juveniles (Pala, 1970, Koya et al., 2003). Therefore an ability to predict embryonic development and clutch size assists in determining the readiness of the females for hormone administration and for determining the number of females required to generate given number of progeny respectively. Such an ability to predict saves time, energy and cost associated with mass production (infrastructure and labour) of feminized *G. holbrooki* since hormone can be administered simultaneously in an organised fashion on a large group of gravid females and the treatment completed within a more defined window of time. In addition, any bias associated with unsynchronised treatment is also mitigated effectively.

One of the interesting observations made while studying the gravid spot relationship and embryonic development was the occurrence of superfetation in *G. holbrooki* embryos.

Superfetation i.e. the presence of more than one clutch of developing embryos in a female at the same time can occur under favourable environmental or special laboratory conditions such as constant lighting and unlimited food supply (Scrimshaw, 1944). The same condition was also observed in the embryos of hormone treated and control females in chapter three and four (Table 3.2, chapter three; Table 4.2, chapter four). This confirms that superfetation occurred commonly in captive *G. holbrooki* compared to those in the wild (Keane and Neira, 2004). Further studies are recommended not only to understand the mechanism but also to identify factors causing superfetation and its function in *G. holbrooki* reproduction strategy.

6.1.2. Gestation and parturition behaviour of *G. holbrooki* in captivity

An experiment was carried out to observe the gestation and parturition behaviour of *G. holbrooki* under different rearing temperatures while testing the consistency of the gravid spot as a marker to predict reproductive development and output. The experiment not only verified the use and effectiveness of the gravid spot as a predictive tool, but importantly, more accurate gestation period and timing of parturition could be documented. Both types of information assisted in optimising resources for fish rearing and served as an effective tool for comparing hormone effects in chapter three, four and five. The information on the influence of temperature on the duration of the gestation period helped in the setting of a consistent rearing temperature throughout all experiments in the three chapters thus facilitating direct comparison of the effects across experiments.

By knowing the diel-timing of parturition, associated manual observation in subsequent experiments could be better targeted and planned. For example, this assisted in targeting the

collection of newly parturated individuals to a window of two hours in the morning, ensuring that the administration of hormone to newborn juveniles was synchronised whilst optimising the number of progeny subjected to treatment. This knowledge also helped in safeguarding most newborn from being cannibalised by the mothers by transferring them to a different rearing tank as soon as the clutch was delivered. Although a breeding trap was fitted in each tank, it does not guarantee the safety of the newborn juveniles. Our earlier observation has shown that there is a likelihood that newborn juveniles swimming back into the mother's compartment of the breeding trap may result in cannibalism by the mother if they are left for a significant period in the parturition tank. In summary knowing the timing of parturition helped to schedule manual checks at the appropriate times of the day, whilst improving the synchronisation of treatment and scheduling observations.

6.2. Sex reversal of *G. holbrooki*

Chapter three and four of this study investigated the efficacy of two estrogens, diethylstilbestrol (DES) and estradiol (E2) in feminizing *G. holbrooki*. Feminisation rather than masculinisation of *G. holbrooki* was the primary focus as it has been suggested that this species has a XX-XY sex determination system (Angus, 1989a, Angus, 1989b, Horth, 2006, Horth et al., 2013). Generation of homogametic carrier males with female phenotype (i.e female with YY genotype) is central to the development of the Trojan Y pest control option (Gutierrez and Teem, 2006). Although no evidence of chromosomal heteromorphy has been found in *G. holbrooki*, recent studies have also indicated that this species possesses the XX-XY sex determination system based on the sex-linked inheritance of melanistic pigmentation in males (Horth, 2006, Horth et al., 2013).

The decision to test the effectiveness of DES to feminize *G. holbrooki* was made based on its recognition as one of the most potent estrogen besides 17α-ethynylestradiol (Pandian and Sheela, 1995, Piferrer, 2001). When the unexpected paradoxical effect of DES was observed in the sex reversal experiments (chapter three), E2 was then chosen and tested on *G. holbrooki* since it is known to be the most potent estrogen among other natural estrogenic compounds such as estrone (E1) and estriol (E3) (Pandian and Sheela, 1995, Piferrer, 2001). Furthermore, E2 has been successfully used in the feminization of other livebearing species such as guppy, black molly and *G. affinis* (Kavumpurath and Pandian, 1993b, George and Pandian, 1995, Senior, 2013).

In the present study, both hormones were administered via ingestion using food as the carrier. Although hormone administration via immersion is much cheaper (Pandian and Kirankumar, 2003), the efficient way of administering the hormones to the embryos of livebearers is through ingestion of the hormone by gravid females as demonstrated in guppies (Kavumpurath and Pandian, 1992, Kavumpurath and Pandian, 1993b). Moreover, observation on *G. affinis* sex reversal found that the survival rates of newborn juveniles immersed in both estrogen and androgen were low despite having a low stocking density (Senior, 2013). In addition, hormone administration does not seem to affect food intake since the intake level of controls and treated fish were comparable (Appendix 3 and 4) thus ensuring that the sex reversal experiments were not jeopardised which might occurred if the treated fish did not consume the hormone enriched food.

The sex labile period and hence window of hormone treatment in this species appears to coincide with final embryonic development extending into the juvenile phase (Pala, 1970, Koya et al.,

2003), therefore the efficacy of the feminizing agents was tested at two different life stages—embryonic stage via gravid female and newborn juveniles (chapter 3 and 4). However, observation on black molly showed that the gravid female aborts the pregnancy during treatment (George and Pandian, 1995) while treatment on newborn juveniles of *G. affinis* showed a low survival rate (Senior, 2013). This prompted parallel testing of the efficacy of treating the two life stages concurrently to avoid loss of time, to optimise resources and reuse of animals. Interestingly, both life stages were susceptible to the sex reversal albeit DES resulted in paradoxical effects. The findings of this study are similar to those observed in guppy (Kavumpurath and Pandian, 1993b) where feminization can be induced during both life stages (embryonic and newborn juveniles).

6.2.1. Feminization vs paradoxical masculinization and its associated impacts on *G. holbrooki* reproduction

This study showed that E2 is an excellent feminizing agent compared to DES. The estradiol treatment successfully produced 100% phenotypic females via E2 treatment at both the embryonic stage and newborn juveniles. Evaluation of the reproductive fitness of E2 treated fish (chapter five) also showed that E2 has a low impact on *G. holbrooki* reproduction thus further endorsing E2 as an effective feminizing agent for this species. Unexpectedly, a rare paradoxical masculinization effect was observed in all fish treated with DES (chapter three) where all the fish possessed an under-developed gonopodium. Gonadal atrophy was also observed in all treated gravid females at the end of the 30 day treatment period despite most of the DES treated fish being able to parturate for the second consecutive time. Observation on fish exposed to DES at 365 days after parturition (DAP) revealed that none had a gravid spot, but instead had under-

developed gonopodia and they did not show any sign of mating when paired with normal females. Lack of feminisation as well as loss of reproductive fitness (inability to mate) of the treated individuals indicates that DES is unsuitable or less suitable to be used for manipulation of sex in *G. holbrooki,* at the administered doses.

Although DES has been successfully used in other fish species, its usage in *G. holbrooki* caused the rare estrogen-induced paradoxical masculinization effect that has never been reported to occur in any fish. It is perplexing why paradoxical masculinization only occurred in DES treated fish and not in fish treated with E2 whereas the latter was also reported to induce paradoxical masculinization in turtles (Warner et al., 2014). However, it is hypothesized (chapter three) that DES disrupts the mechanism of aromatase gene expression that regulates the biosynthesis of estrogen from androgen in the gonad. This disruption potentially leads to the accumulation of androgen thus triggering the masculinization.

Another possibility for the paradoxical masculinization is that the DES might have an adverse effect on the mitotic activity of the germ cell in undifferentiated gonad of *G. holbrooki* as observed in medaka (*Oryzias latipes*) (Paul-Prasanth et al., 2011). The inhibition or disruption of germ-cell mitosis activity caused by biotic or abiotic factors can change the course of sexual differentiation from female to male (Martínez et al., 2014). A third possibility is that estrogen synthesis in the gonad of treated mothers was supressed leading to testosterone accumulation (Fig. 6.1A) and hence paradoxical sex reversal of embryos and gonadal atrophy in treated females following parturition. Unfortunately, the mechanism underlying the adverse effect of DES on the mitotic germ cell proliferation in this species is as yet undocumented. It is also

possible that this synthetic non-steroid EDC might have some genotoxic effect on *G. holbrooki* during its early development that warrants further investigation. Looking at the effects DES had on treated fish even after 11 to 12 months treatment was ceased (under-developed gonopodium and no sign of mating) it is possible that DES might have affected mitotic activity during early *G. holbrooki* development causing adverse effects observed in treated fish even at adulthood. In contrast, E2 is a natural steroid that is also synthesized in the gonad; the ex-vivo administration of E2 might have only had compensatory effects without altering any other pathways such as the germ cell proliferation during early gonad differentiation (Fig. 6.1B).

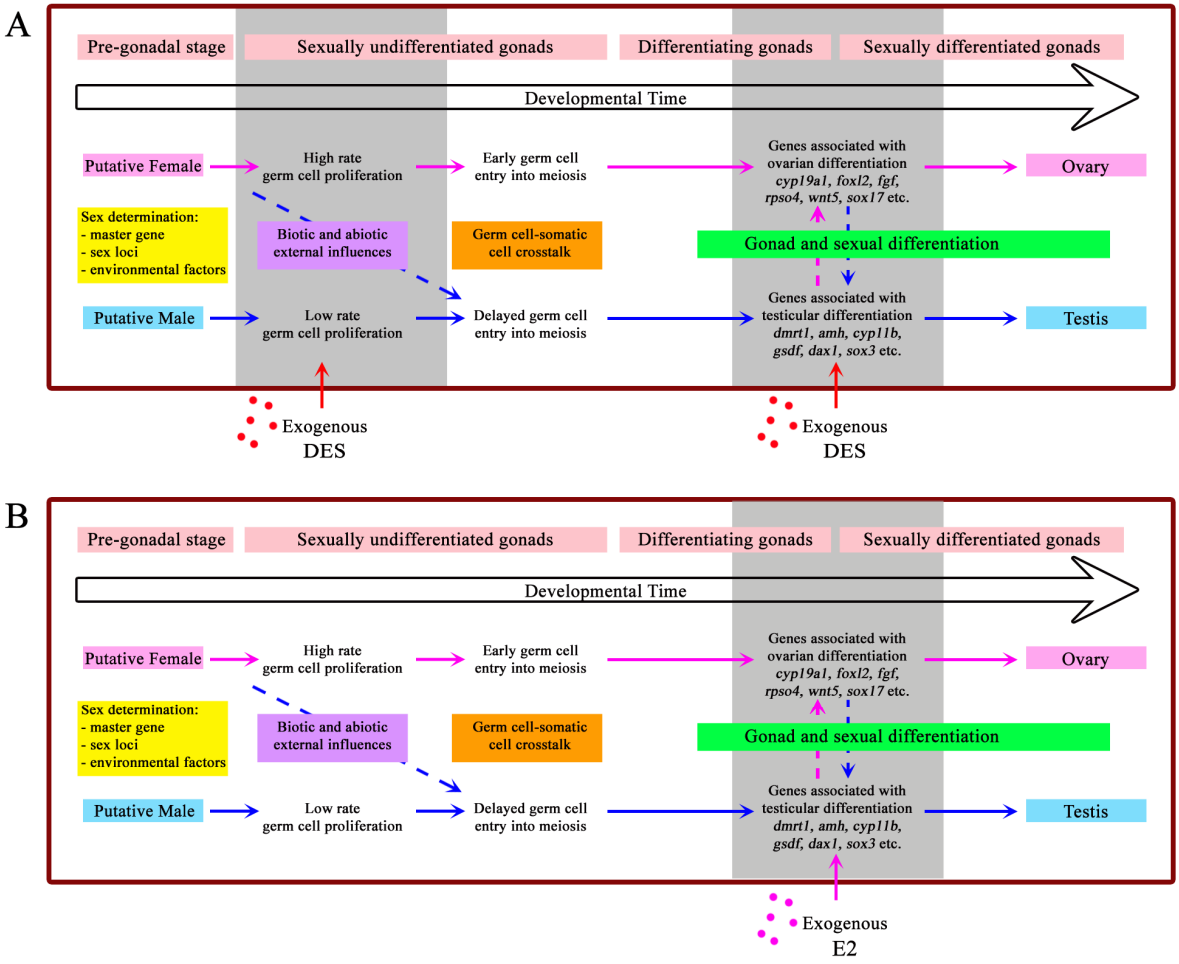

Fig. 6.1. Major events leading to gonad differentiation in male and female fish (adapted from Martínez et al., 2014) and sites of exogenous hormone action/endocrine disruption. (A) Exogenous DES treatment is suggested to affect the germ cell mitotic activity in undifferentiated gonad and steroidogenesis during gonad differentiation (grey shaded areas; red arrows) leading to the paradoxical masculinization effect observed (chapter three). (B) In contrast exogenous E2 potentially only interferes with the gonad differentiation mechanism without affecting germ cell proliferation during early female gonad differentiation (grey shade area; pink arrow).

6.2.2. Survival rates and clutch size

The observed mean survival rates (MSR) of the treated fish are lower compared to controls as well as those of DES and E2 treated fish in guppy (Kavumpurath and Pandian, 1993b) and molly (George and Pandian, 1995). This could be attributed to the toxicity of the respective estrogens that are species specific. Compared to other sex reversal studies on guppy and black molly, the survival rates of treated fish (DES and E2) and controls are quite low in this study. Stocking density was identified as a possible cause of the low survival aside from possible toxicity of the hormone itself. This inference is supported in chapter five where high survival rates were observed in the progenies of the treated fish which were reared at a low stocking density (maximum five fish per litre). Aggressive behaviour by larger and dominant fish towards smaller fish (chapter five) might also contribute to the low survival rate especially at high densities. Further work is needed to verify this density-dependent survival as this will be critical for the mass propagation of this fish in captivity for TSC purposes.

A distinct clutch size difference was observed between parturating gravid females between experimental chapters. The gravid females used in chapter four produced a bigger clutch size (an average of 52 progenies) compared to those in chapter two (average of 12 progenies), chapter three (an average of 13 progenies) and chapter five (and average of three progenies). This condition was unlikely caused by the hormone treatment as they were not significantly different from the respective controls. Also fertilization in the treated individuals occurred before hormone was administered to the gravid females. It is also unlikely the hormone caused the death of the embryo thus reducing the clutch size since the respective controls were not significantly different. For females in chapter five, the small size (length) of gravid females (18.0 – 28.0 mm

TL) was identified as a contributing factor to the small clutch size. However, the size of females used in chapter three and four was within the same range (31.0-46.0 mm TL) eliminating size as the main reason for the observed differences in clutch size between both experimental chapters (chapter three and four).

The stark contrast in clutch sizes observed between those presented in chapters three and four may be attributed to the duration they were reared in captivity prior to the experiments. The gravid females used in chapter two and three were maintained in captive conditions for at least two months before they were used in experiments. In contrast, gravid females in chapter four were collected from the Tamar Island Wetland Reserve (TIWR) a week prior to the sex reversal experiments. Fish reared in captive conditions especially over a longer period may be prone to stress due to handling and rearing environment (temperature, photoperiod, food) which may directly affect reproduction including fecundity (Pankhurst and Van Der Kraak, 1997, Schreck et al., 2001). However, no reports can be found on the effect of stress on the clutch size of livebearers. Furthermore, the nutrition of the commercial food fed to the gravid females (Appendix 6) might be suboptimal thus leading to the small clutch size. There are no reports available to compare the clutch size of *G. holbrooki* in the wild and in captivity since there is lack of research interest on issues related to the propagation (i.e. nutrition requirements) of this economically insignificant species in captivity. Nevertheless, it is clear that the reduction of fecundity will directly affect clutch size since the number of mature eggs to be fertilized will be reduced when fecundity is low. It is recommended that future studies use newly collected fish from the wild to increase the production of progeny.

6.3. Implication of this study and the way forward

This study has successfully demonstrated the feminization of *G. holbrooki* and that the reproductive fitness of treated fish was comparable to controls (Fig. 6.2), paving the way for developing a Trojan chromosome control option for this pest fish. However, the sex marker of *G. holbrooki* must first be developed since identification of female carrying male genotype (sex reversed fish) is crucial in the development and efficient deployment of Trojan carrier females. Although progeny testing can be done to identify the genotype it is not feasible for large scale production of Trojan carriers since it takes time and requires large scale infrastructure to accommodate the breeding and rearing of feminized fish (Dunham, 2011). Instead, development of a sex marker will circumvent the need for long progeny testing and ensures accurate and fast identification of genetic sex as is possible in medaka (Matsuda et al., 2002; Patil and Hinze, 2008) and *G. affinis* (Senior, 2013, Lamatsch et al., 2015).

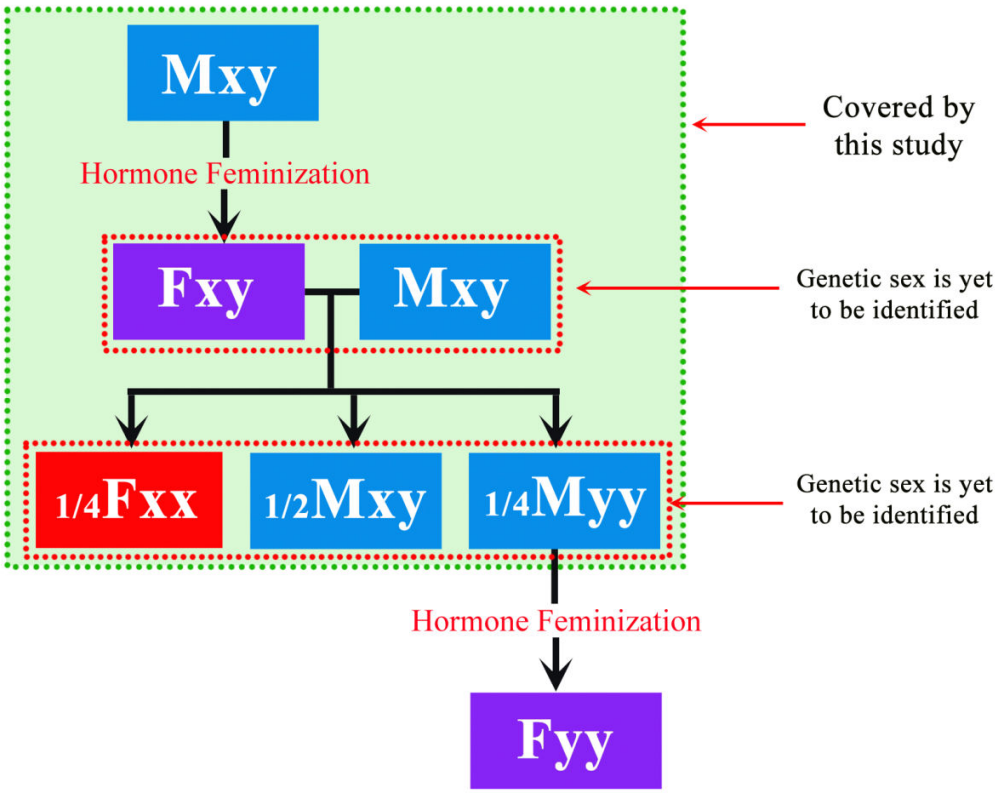

Fig. 6.2. Schematic showing steps involved in the production of Trojan female fish (YY chromosome carrier female), via hormone sex reversal (Gutierrez and Teem, 2006, Cotton and Wedekind, 2007). Green coloured box represents the steps accomplished in this study. The genetic sex of treated fish and its progenies are yet to be determined (red dotted box).

In order to ensure the effectiveness of the TSC, further observations are needed to determine the effects of E2 on the clutch size of *G. holbrooki*. The behaviour and interaction between sex reversed and normal fish must also be addressed since very few studies have been reported (Senior et al., 2012). Studies on behavioural traits could focus on the male mating preference between normal females and feminized fish plus the interaction between the latter two. The only available information on the interaction of normal and fully sex reversed fish was in *G. affinis* where the study reported that normal females displayed aggressive behaviour towards sex reversed females due to size differences (Senior, 2013). Any adverse interaction between normal and sex reversed fish could compromise the control and eradication effort therefore all this information needs to be analysed before the TSC strategy can be applied at large scale in the field. It is suggested that research on behaviour traits is combined/conducted together with the density-dependent survival.

Throughout this study, several issues and knowledge gaps on *G. holbrooki* reproduction, captive propagation and behaviour were identified and highlighted. Issues that require special attention include the mysterious mechanism of internal fertilization of oocytes by stored sperm; gravid spot melanisation and the rare paradoxical masculinization effect on DES treated fish. More research and experimentation are needed to solve the issues raised in this study to help the understanding and advancement of knowledge on the unique reproductive biology of this livebearer.

6.4. Concluding remarks

In conclusion, the main objective of this study was to demonstrate feminization of *G. holbrooki*, which was successfully achieved. The findings of this study are novel and have never been reported in *G. holbrooki*. The findings are beneficial not only for the eradication and control effort of this species but also for other studies involving the biology of livebearers and impact of pollution on aquatic fauna. Importantly, this study has opened a path towards the development and application of the TSC strategy to control and eradicate this species. Focus and effort can now be directed towards the production of Trojan chromosome carrier females in *G. holbrooki*.

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Appendix 1

List of articles and publications related/discussing the Trojan Sex Chromosome Strategy.

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Appendix 2

Table A1. Summary of multiple regression analysis

Variable	B	SE_B	β
Intercept	1.835	0.704	
Intensity	-0.85	0.317	-0.365*
Fish Length	0.196	0.084	0.295*
Spot Size	3.549	3.130	0.155*

Note: *$p<0.05$, B= unstandardized regression coefficient; SE_B= Standard error of the coefficient; β= standardized coefficient

Appendix 3

Quantity of DES delivered to each treated fish at the end of the 30 days treatment period.

Table A2. Average total quantity of food and DES (mean ±SD) delivered to each *G. holbrooki* at the end of the 30 days treatment period.

Experiment	DES Dose	Average total quantity of food given (mg)*	Average total quantity of DES delivered (mg)
Experiment 1: Treatment on embryo via ingestion by gravid females	C1 – without ethanol	298.84 ± 3.434	N/A
	C2 – with 70% ethanol	300.46 ± 2.051	N/A
	T1 - 20 mg/kg feed	298.32 ± 5.027	0.0059 ± 0.0001
	T2 - 40 mg/kg feed	300.24 ± 3.894	0.0120 ± 0.00015
	T3 - 60 mg/kg feed	298.02 ± 4.696	0.0179 ± 0.00027
	T4 - 80 mg/kg feed	298.94 ± 3.732	0.0238 ± 0.00037
	T5 - 100 mg/kg feed	298.80 ± 4.111	0.0298 ± 0.00037
Experiment 2: Treatment on newborn juveniles	C1 – without ethanol	12.66 ± 0.531	N/A
	C2 – with 70% ethanol	12.42 ± 0.553	N/A
	T1 - 20 mg/kg feed	12.92 ± 1.269	0.000258 ± 0.000025
	T2 - 40 mg/kg feed	12.70 ± 1.148	0.000508 ± 0.000046
	T3 - 60 mg/kg feed	12.84 ± 0.673	0.000770 ± 0.000040
	T4 - 80 mg/kg feed	13.44 ± 0.795	0.001075 ± 0.000064
	T5 - 100 mg/kg feed	12.32 ± 0.952	0.001232 ± 0.000095

*Total food given to all fish throughout the treatment duration divided by the number of fish per treatment.'

Appendix 4

Quantity of E2 delivered to each treated fish at the end of the 30 days treatment period.

Table A3. Average total quantity of food and E2 (mean ±SD) delivered to each *G. holbrooki* at the end of the 30 days treatment period.

Experiment	E2 Dose	Average total quantity of food given (mg)*	Average total quantity of E2 delivered (mg)
Experiment 1: Treatment on embryo via ingestion by gravid females	C1 – without ethanol	300.22 ± 3.384	N/A
	C2 – with 70% ethanol	300.14 ± 1.797	N/A
	T1 - 50 mg/kg feed	301.12 ± 1.714	0.0151 ± 0.000086
	T2 - 100 mg/kg feed	298.46 ± 4.275	0.0298 ± 0.000428
	T3 - 200 mg/kg feed	297.34 ± 5.131	0.0595 ± 0.001026
	T4 - 300 mg/kg feed	299.02 ± 3.737	0.0897 ± 0.001121
	T5 - 400 mg/kg feed	298.98 ± 5.251	0.1196 ± 0.002100
Experiment 2: Treatment on newborn juveniles	C1 – without ethanol	12.94 ± 0.913	N/A
	C2 – with 70% ethanol	12.86 ± 0.796	N/A
	T1 - 50 mg/kg feed	12.68 ± 1.303	0.00063 ± 0.000065
	T2 - 100 mg/kg feed	12.80 ± 0.682	0.00128 ± 0.000068
	T3 - 200 mg/kg feed	12.90 ± 1.113	0.00258 ± 0.000223
	T4 - 300 mg/kg feed	13.26 ± 1.318	0.00398 ± 0.000395
	T5 - 400 mg/kg feed	13.32 ± 0.937	0.00533 ± 0.000375

*Total food given to all fish throughout the treatment duration divided by the number of fish per treatment.'

Appendix 5

Analysis of size difference between E2 exposed fish, control females and males.

Methodology:

Size (total length) of all the fish (E2 treated fish = 87; control females = 47; males = 50) were measured to the nearest cm at maturation (70-90 days after parturition (DAP). Data was analysed using Welch's ANOVA and Tukey-Kramer post-hoc test to find any significant differences between the groups.

Results:

Maximum size of E2 exposed fish and control females were both 2.8 cm while it was 2.51 for male fish. The smallest size of E2 treated fish and control females was 1.83 and 1.80 cm respectively. In males, smallest size observed was 1.61 cm. Statistical analysis found a significant difference ($F=16.682$; df: 2, 182; $P<0.05$) between the three fish groups however Tukey-Kramer post hoc test showed that there was no significant difference between the size of E2 treated fish and control females (Figure A).

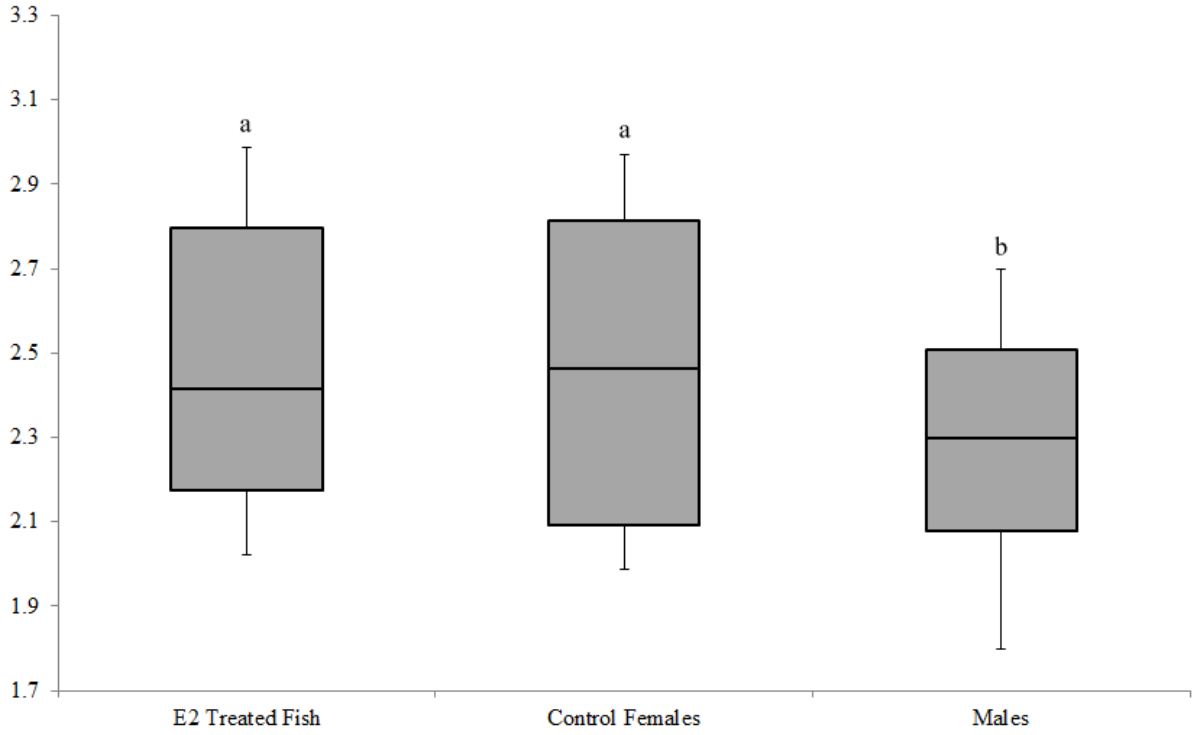

Fig. A1. Size of E2 treated fish, control females and males at maturation (70-90 DAP). Groups with the same superscript were not significantly different from each other (P>0.05).

Appendix 6

Commercial fish food used in this study.

Fig. A2. Commercial fish food used throughout the experiment. (A) Tetramin® Tropical Granules and (B) Hikari® Tropical micropellets.

Nutrient	Tetramin® Tropical granules	Hikari® Tropical micro-pellets
Crude Protein	min. 46.0%	min. 43 %
Crude Fat	min. 7.0%	min. 7 %
Crude Fiber	max. 2.0%	max. 7 %
Moisture	max. 8.0%	max. 10 %
Ash	N/A	max. 17 %
Phosphorus	min.1.4%	min. 1.1 %